Volume 49 NOVEMBER, 1951 Number 5

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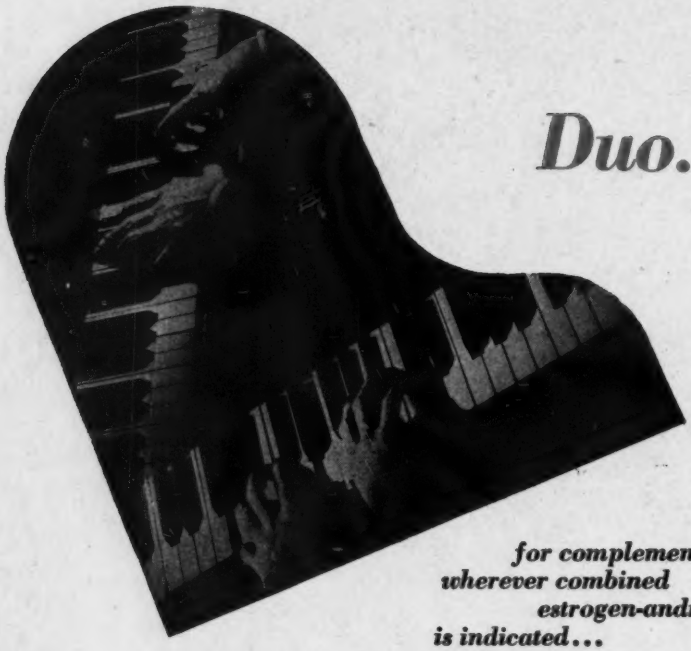

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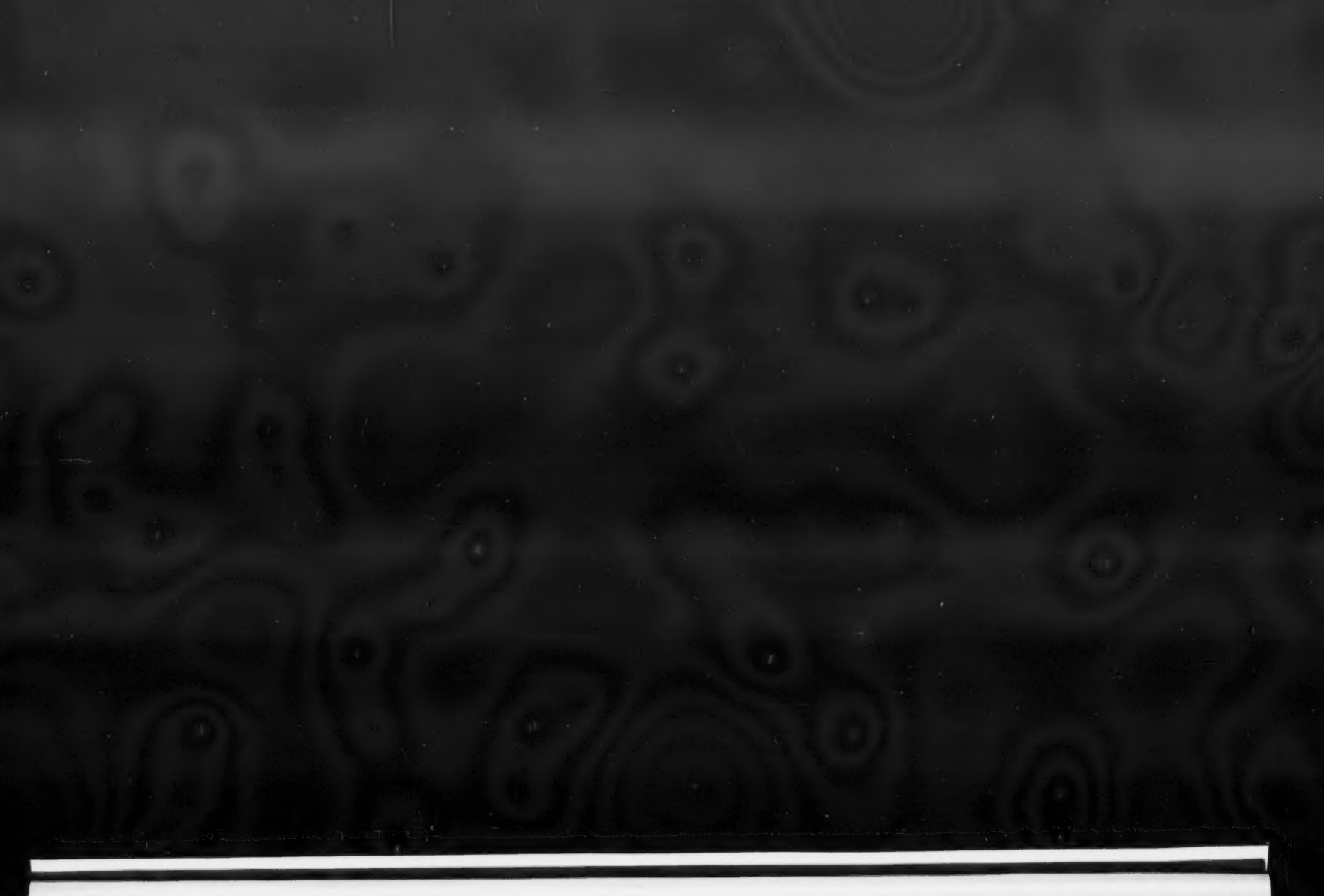

ENDOCRINOLOGY

VOLUME 49 NOVEMBER, 1951 NUMBER 5

EFFECTS OF ESTROGEN AND THYROXINE UPON PLASMA PROTEINS AND BLOOD VOLUME IN THE FOWL*

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COMMON *et al.* (1947 and 1948) and Fleischmann and Fried (1945) demonstrated that estrogen administration to chickens increased the serum calcium, phosphoproteins, lipids, liver fat, and liver protein, but these increases were prevented when thyroxine was administered with the estrogen. McDonald *et al.* (1945) reported similar results in the pigeon.

None of these workers, however, studied the effects of the hormones on the individual plasma protein components, albumin and globulin. Mandel *et al.* (1947) showed that in the pigeon the increase in total plasma proteins resulting from estrogen administration was mainly in the albumin fraction.

Common *et al.* also reported that crude blood volume in the fowl (determined by the exsanguination technique) was increased by estrogens. It is known that this technique is subject to great error.

The object of this study was to determine the effects of estrogen and thyroxine upon plasma albumin and globulin and blood volume of the fowl.

EXPERIMENTAL PROCEDURE

Total plasma proteins and albumin were determined by the biuret method of Gornall *et al.* (1949). Globulin was determined indirectly. These workers compared the biuret method with the micro-Kjeldahl method on rat serum and found the two methods to agree within 0.1 gram per cent. They used 0.5 ml. serum or plasma, and determined the total proteins and albumin with the Elvelyn photoelectric colorimeter. In this study, 1 ml. of plasma and the Klett-Summerson colorimeter were used. Comparison of the micro-Kjeldahl with this method for chicken blood showed agreement within 0.08 per cent.

Received for publication June 14, 1951.

* Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University—the State University of New Jersey, Department of Poultry Husbandry. Presented at the meetings of the Federation of American Societies for Experimental Biology (May, 1951).

Plasma volume was determined as follows: 1 ml. of T-1824 dye (2.5 mgs.) was injected intravenously, and after a mixing time of 3 minutes, blood samples were taken from the heart. Pino and Weiss (1950) have shown that even 2 minutes is usually adequate for complete mixing in the chicken. Concentration of the dye in the plasma and standards was determined colorimetrically. Wintrobe tubes were used for hematocrit.

RESULTS

Estrogen and plasma proteins.—McDonald and Riddle (1945) showed that during the laying cycle, total plasma proteins were elevated in the pigeon, suggesting that the estrogen produced during maturation of the

TABLE 1. EFFECTS OF 0.02 PER CENT DIENESTROL DIACETATE IN FEED FOR 4 WEEKS, THEN 0.04 PER CENT FOR 2 WEEKS. TOTAL OF 9 WHITE LEGHORN FEMALES, DATA PAIRED AND DIFFERENCES TREATED AS MEANS

Groups	Means			A/G
	Total protein gms./100 ml.	Albumin gms./100 ml.	Globolin gms./100 ml.	
1. Control	5.14	1.94	3.20	0.60
2. Estrogen 0.02 per cent	5.50	2.11	3.39	0.61
3. Estrogen 0.04 per cent	8.40	3.20	5.20	0.61
Error of difference				
1–2	0.28	0.116	0.19	
1–3	0.32	0.187	0.24	
P values 1–2	0.3	0.4	0.1	
1–3	<0.001	<0.001	<0.001	

follicles and ovulation accounted for the rise. Sturkie and Newman (1951) found, contrarily, no significant difference in the plasma proteins of laying and non-laying hens, but the A/G ratio was lower in non-laying birds. The data of Rochlina (1934) suggested that the plasma proteins were lower in laying hens. That the plasma proteins of the male fowl are significantly lower than the female was shown by Rochlina and by Sturkie and Newman. Thus, it would appear that in the hen, the normal level of circulating estrogen is not an important factor in the control of plasma proteins, and the amount of estrogen required to elevate the plasma proteins is relatively high, as will be shown.

The estrogen used was dienestrol diacetate. Previous workers (Lorenz, 1945, Lorenz and Bachman, 1947, and others) have demonstrated that dienestrol when administered orally at the level of 0.01 per cent in the feed produced fat deposition and lipemia within 2 to 4 weeks.

In the first experiment, non-laying White Leghorn hens were fed 0.02 per cent dienestrol diacetate (20 mg. per day per bird) in the feed for 32 days and then at the 0.04 per cent level for 14 days. Plasma proteins were determined before treatment and after administration of the hormone at the

above levels. The same birds were used and the values were paired. The data are presented in Table 1. It is apparent that dienestrol at the 0.02 level did not significantly increase plasma proteins or affect the A/G ratio. When the level was increased to 0.04 per cent, all birds showed an increase in plasma proteins within 7 days, and within 14 days the increase was 63 per cent and highly significant. The A/G ratio, however, was not changed significantly.

Some of these birds were then returned to 0.02 per cent dienestrol and blood samples taken again after 2 weeks. In every case plasma proteins dropped back to the levels shown before 0.04 per cent dienestrol was administered.

Estrogen and blood volume.—Another group of laying White Leghorn hens were used to determine the effects of the estrogen on total plasma proteins, plasma volume and hematocrit. Determinations were made before

TABLE 2. EFFECTS OF DIENESTROL DIACETATE ON TOTAL PLASMA PROTEINS, BLOOD VOLUME AND HEMATOCRIT. ESTROGEN ADMINISTERED IN FEED (0.04 PER CENT) FOR 32 DAYS, AND /OR INJECTED (30 MGS.). VALUES PAIRED AND DIFFERENCES TREATED AS A MEANS

	No. birds	Means		Error of difference	P values
		Controls	Treated		
Total protein gms./100 ml.	12	5.04	8.12	0.98	0.10
Plasma volume (cc.)	12	97.66	98.95	2.18	0.60
Cell volume gms./100 ml.	8	26.52	23.05	1.90	0.10

the brids were treated, and then some of the birds were fed 0.04 per cent of the estrogen in the feed for 32 days and some were injected with the estrogen at intervals of 2 weeks (total dosage 30 mgs.). Since there was no difference in the results with these treatments, the results were pooled (Table 2). Here, as was shown in Table 1, total plasma proteins were increased significantly and to about the same extent (61 per cent). Albumin and globulin were determined on 5 of the control and treated birds, and the A/G ratio, as shown in the previous experiment, was not altered appreciably by the estrogen. The ratio was higher for the controls and the treated birds, 0.84 and 0.89 respectively, than for the non-laying birds of the first experiment.

The estrogen had no significant effect upon plasma volume and hematocrit.

Effects of thyroxine on hyperproteinemia induced by estrogen.—In a third experiment the laying hens which were treated with the estrogen for 32 days (Table 2) continued to receive it in addition to 1 mg. of D,L-thyroxine per bird per day for 7 to 10 days. At the end of this period blood samples were taken and determinations were made on plasma albumin and globu-

lin, plasma volume, and hematocrit on 9 of the original 12 birds (Table 3). The thyroxine not only prevented the usual hyperproteinemic response to estrogen, but depressed the total plasma proteins (4.62 gms. per cent) below the control level (5.45). The decrease in albumin below the control level, but not globulin, was statistically significant. The A/G ratio of the thyroxine-treated birds was 0.74, as compared to 0.85 for the controls, but this difference was not statistically significant.

Three of the birds on the estrogen-thyroxine treatment continued to recieve estrogen but not thyroxine, and within 14 days total plasma proteins were elevated again to 8.04 gms. per cent.

TABLE 3. EFFECTS OF DIENESTROL DIACETATE AND THYROXINE UPON PLASMA PROTEINS (GMS. PER CENT) AND BLOOD VOLUME OF LAYING HENS. ESTROGEN ADMINISTERED FOR 32 DAYS (0.04 PER CENT IN FEED OR INJECTIONS) UNTIL PLASMA PROTEINS WERE ELEVATED (SAME BIRDS AS USED IN EXPERIMENT 2), THEN ESTROGEN TREATMENT CONTINUED, PLUS 1 MG. OF D,L-THYROXINE PER BIRD PER DAY FOR 7 TO 10 DAYS. VALUES PAIRED AND DIFFERENCES TREATED AS MEANS

	No. birds	Means		Error of difference	P values
		Control	Estrogen and thyroxine		
Total protein	9	5.45	4.64	0.311	0.05–0.02*
Albumin	9	2.50	1.97	0.225	0.05–0.04
Globulin	9	2.94	2.67	0.187	0.2 –0.1
A/G		0.85	0.74		
Plasma volume (cc.)	8	97.27	95.10	2.600	0.4
Cell volume gm./100 ml.	9	26.15	27.61	1.490	0.3

* Between 0.05 and 0.02.

DISCUSSION

The results of this study show that large doses of estrogen are required to increase the plasma proteins of the chicken, and that the increase in albumin and globulin is approximately the same. This is contrary to the work of Mandel *et al.* (1947) on the pigeon and of Levin and Leathem (1942) on the rat, which showed that the principal increase in plasma proteins following estrogen administration was in the albumin fraction. In these species the A/G ratio is greater than 1, but in the chicken less than 1. Levin and Leathem showed that estrogen caused adrenal enlargement in the intact rat and suggested that the stimulated adrenal released cortical hormones which increased plasma albumin. Estrogen also causes adrenal enlargement in the chicken, according to most investigators (Breneman, 1942; Stamler *et al.*, 1950; Kumarin and Turner, 1949).

The results of this study might suggest, as pointed out by Fleischmann and Fried and by others, that excess thyroxine either inhibits the synthesis of plasma proteins in the liver or increases the oxidative destruction of these constituents. Recently, Common *et al.* (1950) showed that thiouracil also

prevents or inhibits the hyperproteinemia produced by estrogen in the fowl, but does not prevent liver hypertrophy or increase in liver protein as does thyroxine. Contrarily, the thiouracil enhanced the liver hypertrophy and the increase in liver protein. Further work upon the effects of thiouracil on the plasma proteins in the bird is needed.

It is known that large doses of estrogen cause extreme vasodilatation in the bird and this may have accounted for the increased blood volume (determined by exsanguination) reported by Common *et al.*

The work of Levin and Leathem, Levin *et al.* (1942) and Hartman *et al.* (1942) demonstrated that adrenal cortical hormones play an important role in the maintenance of the normal serum albumin level in rats, cats and dogs. Hypophysectomy or adrenalectomy caused a significant decrease in serum albumin and increased globulin, and the level of albumin was restored with cortical extracts. The role of the adrenal in the control of plasma proteins in the chicken has not been investigated.

Levin *et al.* (cited above) and Leathem (1945) also showed that the thyroid activity is associated with the normal level of serum globulin. Thyroidectomy and goitrogens produced significant increases in sreum globulin but little change in albumin in rats and cats. Thyroxine prevented the rise in serum globulin resulting from hypophysectomy or thyroidectomy. The effects of hypophysectomy and thyroidectomy on the plasma proteins in the fowl have not been determined.

SUMMARY AND CONCLUSIONS

Dienestrol diacetate administered to White Leghorn hens at the level of 0.04 per cent in the feed increased total plasma proteins more than 60 per cent, and the increases in albumin and globulin were the same. When the estrogen was fed at the 0.02 level over 32 days, plasma proteins were not elevated significantly.

When D,L-thyroxine and estrogen were administered to hens, the hyperproteinemic response was prevented. At the level administered (1 mg. per bird per day injected) the thyroxine depressed plasma proteins significantly below control levels. The decrease in albumin, but not globulin, was statistically significant.

Neither thyroxine in combination with estrogen nor estrogen alone affected plasma volume or hematocrit significantly.

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EFFECTS OF HYPOPHYSECTOMY, GROWTH HORMONE, THYROXIN AND FOOD INTAKE ON CATHEPSIN ACTIVITY OF RAT TISSUES

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SINCE growth and protein synthesis are intimately related, effects of hormones which alter the rate of growth on tissue enzymes involved in various aspects of protein metabolism are of obvious interest. That cathepsins may be implicated in the synthesis of protein by cells has been suggested, for they are the only known intracellular proteinases possessing the specificity of action required in elaboration of the complex protein molecule (Fruton, 1947). Thus, it appeared of interest to determine whether or not the cathepsin activity of liver, kidney, or spleen of rats changes when large variations in the rate of gain in weight are produced by hypophysectomy, treatment with growth hormone or thyroxin, or control of food intake.

EXPERIMENTAL

In general, the design of experiments was the same as in previous studies (Gaebler and Mathies, 1951). Five series of Sprague-Dawley female rats were used. Series K, L, M, and N each included 4 groups; Series O consisted of 3. The number of rats per group was usually 5, exceptionally 4 to 8. Hypophysectomized animals and normal controls of the same age were purchased from the same supplier.[1] Growth rates of operated animals used in experiments did not exceed those characteristic of hypophysectomized rats (Shaw and Greep, 1949). Details of age, manner of feeding, and treatment are given in the Tables. The diet of Bennett, Li and Laundrie (1948) was used in all experiments.

The lyophilized growth hormone preparation[2] used in this study had an activity of 1740 R.U./gm. (Gaebler and Mathies, 1951), and was injected intraperitoneally as a saline suspension.

At the end of an experiment, the animals were sacrificed, the liver, kidneys and spleen rapidly removed and placed on cracked ice prepared from distilled water. These organs were quickly blotted free of excess moisture, weighed to 2 mg. on a Roller-Smith balance, placed in vials, frozen on dry ice and stored at −18°. Within the next

Received for publication July 5, 1951.

[1] Hormone Assay Laboratories, Chicago.

[2] We are indebted to Parke, Davis & Co. for this preparation supplied through the courtesy of Dr. D. A. McGinty and Mr. L. W. Donaldson. An assay of our own is given in the cited reference.

week, homogenates were prepared (Potter and Elvehjem, 1936) in double distilled water at 0°. These homogenates were frozen on dry ice and stored at −18° for the cathepsin and total nitrogen estimations.

Total nitrogen determinations were carried out in triplicate on pooled homogenate by the procedure of Hiller, Plazin and Van Slyke (1948). Cathepsin activity of the properly diluted homogenates was estimated by the procedure of Anson as given by Northrop *et al.* (1948). The digestion period was 10 or 30 minutes with all results calculated in terms of apparent mg. tyrosine liberated/hour. The Klett-Summerson photoelectric colorimeter (red filter) was used to determine optical densities.

RESULTS

I. *Large Doses of Growth Hormone*

In animals of Series K (Table 1) large doses of growth hormone, together with *ad lib.* feeding, produced rapid gain in weight in the treated hypoph-

TABLE 1. RENAL CATHEPSIN IN HYPOPHYSECTOMIZED AND NORMAL RATS BEFORE AND DURING TREATMENT WITH LARGE DOSES OF GROWTH HORMONE

Experiment	No. of rats per group	Average body wt. at start of treatment in gm.	Average body wt. gain during treatment in gm.	Average daily food intake in gm.	Average terminal organ wt. in gm.	Apparent mg. tyrosine liberated per hour		
						Per gm. fresh tissue	Per mg. total nitrogen	Per 100 gm. body wt.
Series K: The rats were 1 month old at operation. Groups IA & IB were fed *ad lib.*; IIA was pair-fed against IA; IIB against IB. Growth hormone treatment was started 12 days postop.; Groups IB & IIB received 1 mg. growth preparation daily per rat for 20 days, and were sacrificed the day following the last injection.								
I. Hypophysectomized.								
A. Controls	5	84	−0.6	5.4	0.522	15.6±1.8	0.545	9.84±1.99
B. Treated	5	74	72	8.1	0.826	19.2±0.3	0.679	10.8 ±0.66
II. Normals								
A. Controls	5	98	5	5.4	0.771	18.9±1.9	0.640	14.0 ±0.89
B. Treated	5	94	59	8.1	1.012	22.6±1.4	0.785	14.9 ±1.57
Series L: Same conditions as in Series K, except that groups IB, IIA & IIB were pair-fed against group IA.								
I Hypophysectomized.								
A. Controls	8	79	0.5	5.5	0.506	16.8±2.3	0.533*	10.7 ±1.3
B. Treated	5	80	30	5.5	0.661	2.31±3.1	0.746	13.8 ±1.9
II. Normals								
A. Controls	4	92	0	5.5	0.702	19.1±1.1	0.623	14.6 ±0.8
B. Treated	5	99	2	5.5	0.787	20.9±2.6	0.677	16.5 ±3.0

* Data from 5 rats.

ysectomized group and in the normal group pair-fed against it. Renal cathepsin was lower in the hypophysectomized controls than in pair-fed normal controls by all 3 methods of calculation, and was increased in the treated groups when calculated per gram of tissue or per milligram of nitrogen. The rise was not significant in terms of activity per organ per 100 grams of body weight. In spleen and liver (Table 2), the cathepsin values in the hypophysectomized control group of Series K were likewise lower than in the normal controls, but treatment produced no consistent changes.

Animals of Series L (Tables 1 and 2) received the same large doses of growth hormone as those of Series K, but the plan of feeding was different.

The food intake of the other 3 groups was limited to that of the hypophysectomized controls. Under these conditions, only the treated hypophysectomized group gained weight (Table 1). Cathepsin activity in the

TABLE 2. CATHESPINS OF SPLEEN AND LIVER IN HYPOPHYSECTOMIZED AND NORMAL RATS BEFORE AND DURING TREATMENT WITH LARGE DOSES OF GROWTH HORMONE

Experiment	Average terminal organ wt. in gm.	Apparent mg. tyrosine liberated per hour		
		Per gm. fresh tissue	Per mg. total nitrogen	Per 100 gm. body wt.
		Spleen		
Series K: See Table 1 for experimental details.				
I. Hypophysectomized.				
A. Controls	0.168	30.5±1.2	1.10	6.15±0.87
B. Treated	0.434	30.1±0.8	1.04	8.88±0.70
II. Normals.				
A. Controls	0.269	35.5±4.6	1.25	9.20±1.27
B. Treated	0.432	33.5±1.0	1.17	9.43±0.47
Series L: See Table 1 for experimental details.				
I. Hypophysectomized.				
A. Controls	0.150	34.9±2.8	1.15*	6.67±0.90
B. Treated	0.290	36.0±1.9	1.21	9.38±1.05
II. Normals.				
A. Controls	0.244	37.5±2.8	1.23	9.93±0.51
B. Treated	0.228	41.6±1.3	1.35	9.46±0.65
		Liver		
Series K: See Table 1 for experimental details.				
I. Hypophysectomized.				
A. Controls	2.99	13.9±2.0	0.403	49.2±5.2
B. Treated	5.25	12.1±0.95	0.393	43.2±3.9
II. Normals.				
A. Controls	2.79	15.3±1.5	0.407	41.4±1.5
B. Treated	4.59	16.6±3.6	0.456	49.4±8.5
Series L: See Table 1 for experimental details.				
I. Hypophysectomized.				
A. Controls	2.90	11.1±2.0	0.291*	40.4±5.5
B. Treated	3.33	16.1±0.86	0.441	48.7±7.0
II. Normals.				
A. Controls	2.45	13.3±1.7	0.321	35.6±3.6
B. Treated	2.84	17.1±1.4	0.437	48.6±4.9

* Data from 5 rats.

kidney (Table 1) as well as in spleen and liver (Table 2) was again low in hypophysectomized controls as compared with normal controls, and values in both treated groups increased in all 3 organs. In the case of kidney and liver this rise was entirely independent of the method of calculation; in spleen, it was evident in two of the 3 methods.

II. *Small Doses of Growth Hormone*

Animals of Series M (Table 3) were all hypophysectomized and fed *ad lib.* The values for cathepsin in kidney, liver, and spleen were all below those of the normal controls of the preceding Series K and L. In the kidney and spleen, treatment produced no effect; in the liver, the small effect was progressive only in terms of activity per milligram of nitrogen. The amounts of growth hormone administered produced decided increases in body weight (Table 3, footnote).

TABLE 3. TISSUE CATHEPTIC ACTIVITY IN HYPOPHYSECTOMIZED RATS GIVEN SMALL AMOUNTS OF GROWTH PREPARATION

Experiment	Average terminal organ wt. in gm.	Apparent mg. tyrosine liberated per hour		
		Per gm. fresh tissue	Per mg. total nitrogen	Per 100 gm. body wt.
Series M: The rats were 1 month old at operation. *Ad libitum* feeding throughout. Growth hormone treatment was started 17–19 days postop. Each rat received 10 daily injections of the amount of hormone indicated for its group, and was sacrificed on the day following the last injection. All rats used in this experiment were hypophysectomized.				
		Kidney		
0 gamma	0.504	17.9±0.9	0.611	11.4±0.5
20 gamma	0.566	17.4±0.5	0.572	11.9±0.5
40 gamma	0.547	17.2±0.8	0.587	11.5±0.8
80 gamma	0.615	16.8±1.0	0.577	11.3±0.9
		Liver		
0 gamma	3.16	10.2±0.5	0.315	40.5±3.1
20 gamma	3.00	12.7±0.8	0.370	46.1±3.3
40 gamma	3.14	13.0±1.2	0.382	49.8±8.1
80 gamma	3.52	12.7±1.1	0.429	48.8±5.6
		Spleen		
0 gamma	0.161	30.5±1.5	—	6.18±1.09
20 gamma	0.182	30.9±1.7	—	6.88±0.82
40 gamma	0.177	31.3±1.9	—	6.64±1.00
80 gamma	0.216	31.2±0.9	—	7.39±0.90

There were 5 rats per group. The average body weights for the 4 groups at the start of treatment were 80, 75, 69, and 72 gm. respectively; the average weight gains during treatment were −0.6, 7.8, 13.8, and 19.6 gm. respectively.

III. *Thyroxin*

The first plan of feeding, in which the two hypophysectomized groups receive food *ad lib.* and the corresponding normal groups are pair-fed against them, was used again in the case of Series N (Table 4). The hypophysectomized group which was treated with thyroxin did not lose weight, while the normal group treated and fed in the identical manner lost considerable weight. Kidney weight increased in both treated groups, but much more strikingly in the hypophysectomized one. The changes in renal cathepsin cannot be interpreted without further study. Spleen cathepsin (Table 5, Series N) was unaltered by thyroxin in either group, while liver cathepsin changed strikingly in both. The values in liver tissue of the

treated hypophysectomized group not only rose far above those of hypophysectomized controls, but well above those of the normal controls, and the same high values were found in treated normals. This increase was large, and consistent by all methods of calculation.

IV. *Change in Food Intake*

In all preceding experiments, the normal control group was pair-fed against hypophysectomized controls and might, therefore, be considered undernourished. Accordingly, a third plan of feeding was employed in Series O (Tables 4 and 5). One group of normal controls was pair-fed

TABLE 4. EFFECTS OF THYROXINE AND MANNER OF FEEDING ON RENAL CATHEPSIN

Experiment	No. of rats per group	Average body wt. at start of treatment in gm.	Average body wt. change during treatment in gm.	Average daily food intake in gm.	Average terminal kidney wt. in gm.	Apparent mg. tyrosine liberated per hour		
						Per gm. fresh tissue	Per mg. total nitrogen	Per 100 gm. body wt.
Series N: The rats were 1 month old at operation. Groups IA & IB were fed *ad lib.*; IIA was pair-fed against IA IIB ageinst IB. Thyroxine treatment was started 28 days postop.; Groups IB & IIB were given 50 gamma thyroxine daily per rat for 7 days, and were sacrificed the day following the last injection.								
I. Hypophysectomized.								
A. Control	6	73	0.3	4.77	0.476	19.0±1.3	0.635	12.3±0.9
B. Treated	5	74	0.8	4.66	0.716	17.7±1.7	0.613	16.4±1.2
II. Normals.								
A. Control	5	92	3.2	4.77	0.695	17.9±0.8	0.609	13.6±1.2
B. Treated	5	93	−15.6	4.66	0.768	21.1±0.9	0.696	20.9±1.4
Series O: The rats were 1 month old at operation. Group IA was fed *ad lib.*; IIA was pair-fed against IA; IIB was fed *ad lib.* No other treatment employed. All rats were sacrificed at 2 months of age. Body weight and food intake data are for the last 20 days prior to sacrifice.								
I. Hypophysectomized.								
A. Control	4	65	2.0	4.74	0.456	15.9±0.7	0.552	10.8±0.7
II. Normals.								
A. Controls	4	86	−2.0	4.74	0.665	18.7±1.1	0.608	14.9±1.7
B. Controls	4	132	65.0	14.0	1.449	18.9±1.9	0.668	13.8±1.4

against hypophysectomized controls; a second one was fed *ad lib.* The food intake and gain in weight of the 2 normal groups differed immensely (Table 4), but renal cathepsin (Table 4) and liver cathepsin (Table 5) were not increased in the normals which were on a restricted food intake. The low values in kidney and liver tissue of hypophysectomized animals were therefore real. In the spleen (Table 5, Series O) low values per gram of tissue in hypophysectomized controls may have been apparent.

DISCUSSION

Our experiments indicate that values for cathepsins in spleen, kidney, and liver are below normal in hypophysectomized rats, most consistently so in the liver. The effect of thyroxin on hepatic cathepsin is much more striking than that of growth hormone, hence low values in hypophysec-

tomized animals are probably related to deficiency of thyrotrophin rather than somatotrophin. In normal rats, the effect of thyroxin on hepatic cathepsin is equally evident, and has been observed previously (Scoz and De Caro, 1935).

The method of feeding and the dosage of growth hormone appear to be

TABLE 5. EFFECTS OF THYROXINE AND MANNER OF FEEDING ON CATHEPSIN IN SPLEEN AND LIVER

Experiment	Average terminal organ wt. in gm.	Apparent mg. tyrosine liberated per hour		
		Per gm. fresh tissue	Per mg. total nitrogen	Per 100 gm. body wt.
		Spleen		
Series N: See Table 5 for experimental details.				
I. Hypophysectomized.				
A. Control	0.143	31.1 ±2.0		6.13±0.55
B. Treated	0.264	30.9 ±2.2		8.79±1.28
II. Normals.				
A. Control	0.214	37.5 ±7.5		10.4 ±2.0
B. Treated	0.170	38.0 ±2.1		8.25±1.52
Series O: See Table 5 for experimental details.				
I. Hypophysectomized.				
A. Control	0.144	29.2 ±0.6		6.19±0.89
II. Normals.				
A. Control	0.219	33.9 ±2.2		8.78±0.90
B. Control	0.569	29.7 ±1.3		8.77±1.10
		Liver		
Series N: See Table 5 for experimental details.				
I. Hypophysectomized.				
A. Control	3.01	9.85±1.73	0.296	40.0 ±4.7
B. Treated	2.55	21.7 ±1.4	0.615	79.3 ±5.6
II. Normals.				
A. Control	2.73	14.9 ±1.1	0.389	40.0 ±2.9
B. Treated	2.15	23.2 ±2.5	0.631	64.3 ±9.0
Series O: See Table 5 for experimental details.				
I. Hypophysectomized.				
A. Control	2.81	9.14±0.61	0.273	38.1 ±2.0
II. Normals.				
A. Control	2.28	14.5 ±1.9	0.365	39.7 ±7.8
B. Control	8.11	13.2 ±1.8	0.419	53.1 ±6.2

quite important. In Series L, in which the doses of growth hormone were large and all groups received the same amount of food, cathepsins increased in spleen, kidney, and liver of the treated groups, whether hypophysectomized or normal. This consistent effect was not observed in Series K, which received equally large doses of growth hormone, but in which operated and treated groups were fed *ad lib.* and corresponding normal groups

were pair-fed against them. A definite and consistent effect of growth hormone on cathepsin concentration was again absent in Series M (Table 3) which received small doses and food *ad lib.* An increase in cathepsin concentration was therefore observed when doses of growth hormone large enough to have produced maximal gains in weight were given to animals whose diet did not permit such gains to occur.

That the concentration of cathepsin was not notably affected by growth hormone in other experiments must not be regarded as evidence that cathepsins are not involved in induced growth. It would be as erroneous to argue that protein is not involved because its concentration in growing tissue remains fairly constant. In many instances increases in organ weights, as indicated in the tables, were very large, and constancy of concentration indicates proportionate though not unique increase in cathepsin. That the concentration remained reasonably constant during extraordinary changes in organ weights and body weight moreover suggests that cathepsins are important cell components.

Whether the growth hormone stimulates anabolic processes, inhibits catabolic ones, or affects both in such a manner as to make the net effect anabolic, is a very involved question. It is difficult even to determine when the net effect is anabolic. Previous studies in this laboratory (Mathies, Gaebler, and Palm, 1949), (Bartlett and Glynn, 1950), include many instances in which growth hormone increased body weight and diminished liver weight. Thus the net effects of growth hormone on body weight and liver weight are not necessarily, as in the present experiments, both anabolic. The present observations with thyroxin further emphasize this difficulty. In hypophysectomized rats (Table 4, Series N), thyroxin produced non-significant gain in weight; in normal pair-fed ones, a significant loss. The net effect on body weight was catabolic only in the normal group. The effect on organ weights, which would presumably be more relevant when one is considering enzymes of individual organs, is even more confusing. Thyroxin exerted a large net anabolic effect in hypophysectomized rats and a smaller one in normal ones, if one considers kidney weights (Table 4, Series N); a net anabolic effect in hypophysectomized animals, but a net catabolic one in normals, if spleen weights are considered; and a net catabolic effect in both groups on the basis of liver weights (Table 5, Series N). Finally, the fact that thyroxin administration was followed by decreases in liver weights and increases in hepatic cathepsin of both groups cannot be regarded as evidence that a catabolic effect of thyroxin is mediated by cathepsin, for the same findings would support the assumption that the function of cathepsin is anabolic, and that the increase in its concentration is a compensatory phenomenon. We have previously emphasized (Gaebler and Mathies, 1951) that development and involution

proceed simultaneously during growth, whether one considers structures or proteins. The effects of growth hormone cannot be considered as entirely anabolic, or those of thyroxin entirely catabolic. Moreover, changes in enzyme concentrations of individual organs or tissues only become helpful in understanding the mechanism of action of either hormone when they are considered together with other evidence, independently supported.

SUMMARY

Assays of cathepsin in the kidney, spleen, and liver of hypophysectomized rats yield lower values than in normals, particularly in the case of liver. Growth hormone produced a consistent rise in cathepsins of the three organs only when large doses were given to hypophysectomized and normal animals which were pair-fed against hypophysectomized controls, and therefore incapable of a maximal response with respect to gain in weight. Under conditions of *ad lib.* feeding, daily injection for 10 days of 20 to 80 gamma doses of growth hormone having an effect on weight about 38 per cent as great as that of purified hormone did not restore cathepsin values. Daily injection of 50 gammas of thyroxin greatly increased hepatic cathepsins in hypophysectomized as well as in normal rats. The low values in the former animals are thus ascribed to deficiency of thyrotrophin rather than somatotrophin. Cathepsin activity in the three organs examined remains comparatively constant during changes in organ weight which would dilute these enzymes several times if they were inert cell constituents. Problems which arise when one characterizes effects of hormones as anabolic or catabolic and attempts to explain these effects on the basis of changes in tissue enzyme concentration are discussed.

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THE EFFECT OF STRESS UPON THE KETOSTEROID AND NON-CARBONYL LIPOID CONTENT OF THE ADRENAL CORTEX OF THE RAT*

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CHANGES in the chemical content and structural pattern of the adrenal cortex following various forms of stress have been reported (Dalton, 1944; Sayers, 1945; Dempsey, 1946; Deane, 1946; Deane, 1947; Long, 1947; Tepperman, 1947; Greep, 1949; Greep, 1950). The significance of these changes and in particular the relation of the chemical to the structural variations is in some respects obscure. There is general agreement that the rate of ketosteroid secretion and fluctuations in lipoid and ascorbic acid content of the adrenal are involved in the reaction to stress.

In the experiments reported below we present data: (1), comparing the normal adrenal with the adrenal of the animal under stress in content and distribution of ketosteroid, as demonstrated morphologically by a stain which is specific for carbonyl groups (Ashbel, 1949) and in the adrenal has been shown to demonstrate ketosteroid (Seligman, 1951; Ashbel, 1951); (2), on the relationship of non-carbonyl containing lipoid to ketosteroid; (3), on the effect of ACTH on the content and distribution of ketosteroid in the normal adrenal cortex and in the adrenal of animals exposed to stress.

METHOD

Control animals. A determination of the normal distribution of ketosteroid is difficult because of the marked sensitivity of the adrenal to the slightest manipulation and to environmental variations. An arbitrary "norm" was therefore selected, which consisted of data from 30 female rats of the same strain and of approximately the same weight (150–180 gms.), living under identical environmental conditions, receiving the same diet, and sacrificed by a blow on the head.

Data on the effect of stress were obtained by subjecting precisely similar rats to various types of stress prior to sacrifice. The adrenals of "normal" and stressed animals were removed immediately after death and fixed in a 10% solution of neutral formalin for 24 hours or longer. The organs were weighed after fixation and the ratio between body weight and adrenal weight calculated.

Received for publication June 14, 1951.

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The types of stress utilized were (a) intraperitoneal ACTH; (b) exposure to cold; (c) exposure to whole body x-ray irradiation; (d) starvation; (e) hemorrhagic shock.

(a) *Intraperitoneal ACTH.* In the first of two groups of 10 rats, each rat received a single injection of 1.5 mgs. of ACTH intraperitoneally, and was sacrificed after one or six hours. In the second group each rat received a daily intraperitoneal injection of 1.5 mgs. of ACTH for seven days and was then sacrificed.

(b) *Exposure to cold at 6°C.* Four groups of 10 female rats each were exposed in the cold room to a temperature of 6°C for 3 hours, 6 hours, 24 hours and 72 hours respectively. A fifth group of ten was exposed for six hours immediately after one intraperitoneal injection of 1.5 mg. of ACTH. Half the animals in each group were sacrificed immediately afterward; the remainder 6 hours later.

(c) *Whole body x-ray irradiation.* Two groups of 10 animals were exposed to a single dose of 200 r and of 400 r respectively and were sacrificed either immediately or at one, six and 24 hours afterward.

(d) *Prolonged starvation.* Two groups of ten rats were used. One group was starved for 3 days, and the second for 7 days. Water was provided *ad libitum.* The animals were all sacrificed immediately following the cessation of stress.

(e) *Hemorrhagic shock.* Eight rats were put into irreversible hemorrhagic shock and were sacrificed 4–6 hours after the irreversible stage was reached (E. Friedman, to be published).

Staining technic

Frozen sections 7μ thick were cut from the formalin-fixed adrenals and stained by the specific reaction for carbonyl groups, which develops a blue compound in lipoid due to ketonic groups (ketosteroids) (Ashbel, 1949; Seligman, 1951; Ashbel, 1951). At least 30 sections from each adrenal were so prepared and examined in order to correct for possible staining variations within the same gland. The stained sections were then mounted in Kaiser glycerogel medium.

For comparison of the distribution of non-carbonyl lipoid to ketosteroid, a Sudan IV stain of every specimen was also prepared. For a more accurate determination of the possible morphological relationship of ketosteroid and non-carbonyl lipoid, all sections were also stained for both ketosteroid and total lipoid as follows: After the ketosteroid stain was performed, the section was washed for 5 minutes in distilled water, then immersed for 5 minutes in Sudan IV, then washed in 50% alcohol and distilled water respectively for 2 minutes, and mounted in Kaiser glycerogel medium.

Although Sudan IV stained all the lipoid, the blue-stained areas contained both ketosteroid and non-carbonyl lipoid (purple), and the red areas contained only non-carbonyl lipoid.

RESULTS

Gross appearance. The adrenals of both normal and stressed animals were light brown in color. Exposure to stress frequently increased the average ratio of adrenal to body weight, as already reported (Ingle, 1938; Engel, 1943). Occasionally, prolonged exposure to stress produced no change in the ratio. In some instances ulcerations were found on the surface of the gland.

Microscopic findings in the adrenal of the normal female rat (Figure 1). The distribution of ketosteroid and non-carbonyl lipoid in the adrenal of

the normal female rat is as follows: In the glomerulosa the ketosteroid is scant and is distributed in the form of fine granules in the cytoplasm or as perinuclear granules in some cells. Non-carbonyl lipoid predominates and

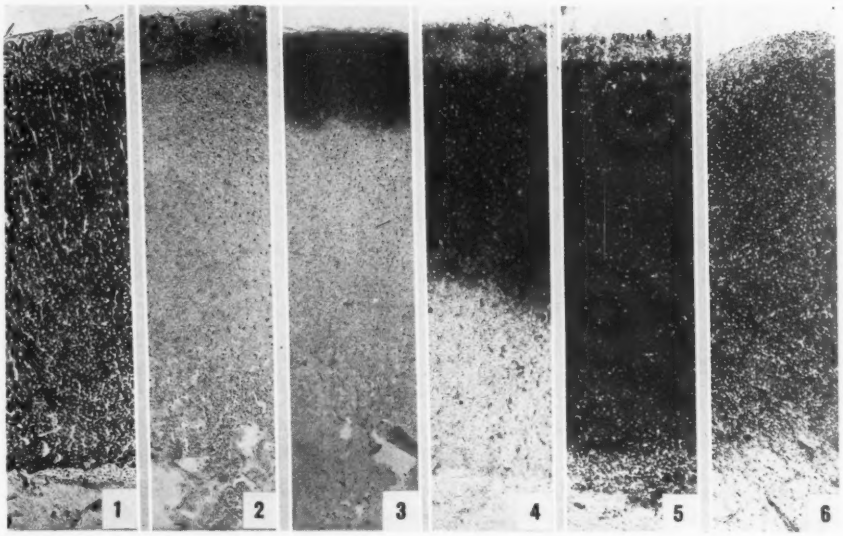

PLATE I. Adrenal cortices of rats receiving ACTH, except Figure 1, which is a normal animal. All sections stained for ketosteroid.

FIG. 1. Ketosteroid distribution in adrenal of normal animal.

FIG. 2. Ketosteroid distribution in adrenal of animal sacrificed 1 hour after ACTH administration. Note excessive depletion of ketosteroid content.

FIG. 3. Ketosteroid distribution in adrenal of animal sacrificed 4 hours after ACTH administration.

FIG. 4. Ketosteroid distribution in animal sacrificed 6 hours after ACTH administration. Note progressive reaccumulation of ketosteroid.

FIG. 5. Ketosteroid distribution in the adrenal of animal sacrificed 24 hours after administration of one single ACTH injection. Note increased accumulation of ketosteroid and the normal pattern of distribution.

FIG. 6. Ketosteroid distribution in adrenal of animal receiving one daily ACTH injection for 7 days. There is no difference from the normal.

is diffusely distributed as fine cytoplasmic droplets. An occasional cell contains both ketosteroid and non-carbonyl lipoid.

The outer third of the fasciculata is rich in ketosteroid, which is diffusely distributed as fine droplets in the cytoplasm. Non-carbonyl lipoid is sparse in this area. In the inner two-thirds of the fasciculata the ketosteroid and non-carbonyl lipoid are present in about equal amounts. The sinusoids are

not congested and the endothelial cells contain the blue stain of the carbonyl reaction.

In the reticularis non-carbonyl lipoid predominates. Small quantities of ketosteroid are present and usually located in the periphery of the cells.

Intraperitoneal injection of 1.5 mgs. of ACTH

Within the first hour following injection there is a severe depletion in ketosteroid (Figure 2). With the exception of the glomerulosa, which is scantily stained, the ketosteroid is limited to the periphery of the outer zone of the fasciculata. The distribution of ketosteroid remains essentially the same in animals sacrificed four hours thereafter (Figure 3). A progressive reaccumulation is evident by the sixth hour when the cortex, with the exception of the reticularis, is stained blue (Figure 4). This staining is still more intense after 24 hours, at which time it has reached the "normal" or a greater than "normal" level (Figure 5). The inverse relationship between ketosteroid and non-carbonyl lipoid is also present in this group.

No significant deviation from the "normal" was observed in 9 of the 10 animals receiving one daily injection of ACTH for a period of 7 days (Figure 6).

Exposure to cold (6°C.) for 3 and for 6 hours. The distribution of ketosteroid in animals exposed to cold for 3 and for 6 hours is markedly different from the "normal."

The glomerulosa shows no change in non-carbonyl lipoid or ketosteroid. The sinusoids of the gland are dilated and congested and their endothelial cells take the ketosteroid stain.

The characteristic change is found in the fasciculata, which shows a *decrease* in ketosteroid, the distribution of which is limited exclusively to a narrow strip 2 to 3 cells in width at the periphery of the outer zone (Figures 7 and 8), where the stain is unevenly distributed in droplets or clumps. The remainder of the fasciculata and the entire reticularis are completely devoid of ketosteroid and present a grayish-white background. Sections stained by the combined method show a sharp decrease of non-carbonyl lipoid in the outer zone of the fasciculata, but the inner zone, which is devoid of ketosteroid, is intensely sudanophilic.

The reticularis shows no ketosteroid and only a sparse distribution of non-carbonyl lipoid.

Exposure to cold for 24 hours. The cytoplasm of the cells of the glomerulosa is loaded with sudanophilic material, but is otherwise unchanged.

The concentration of ketosteroid in the fasciculata is *increased.* The increase is expressed by a widening of the zone containing the blue compound (Figure 9). A few scattered cells contain both ketosteroid and non-carbonyl lipoid, but the majority of the cells are devoid of the latter,

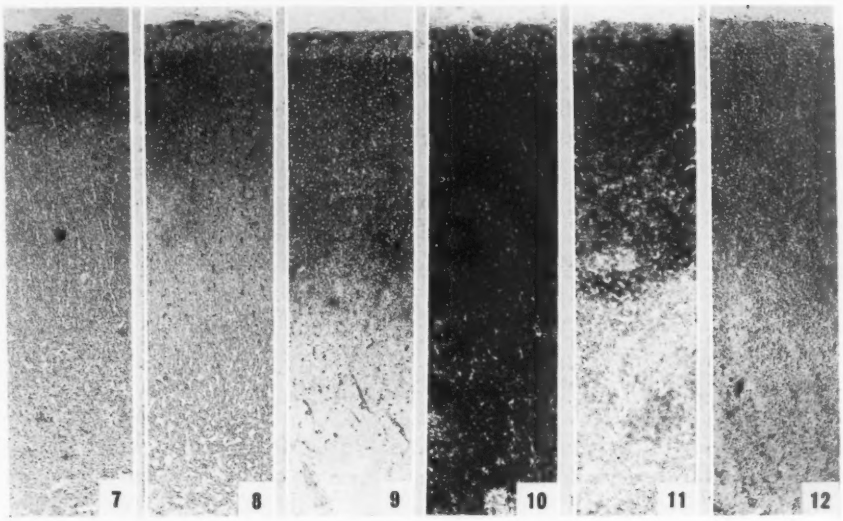

PLATE II. Adrenal cortices of female rats exposed to cold (6°C) and stained for ketosteroid.

FIG. 7. The distribution of ketosteroid in the adrenal of an animal exposed to cold for 3 hours and sacrificed immediately. Note presence of ketosteroid only in a narrow ring at the periphery of the fasciculata.

FIG. 8. The distribution of ketosteroid in the adrenal of animal exposed to cold for 6 hours.

FIG. 9. The adrenal of an animal exposed to cold for 24 hours showing progressive reaccumulation of ketosteroid in the fasciculata.

FIG. 10. The adrenal of an animal exposed to cold for 72 hours showing a pattern of distribution similar to the normal.

FIG. 11. The adrenal of an animal exposed to cold for 3 hours and sacrificed 6 hours after cessation of stress. Note progressive reaccumulation of ketosteroid within this period.

FIG. 12. The adrenal of an animal given ACTH immediately period to exposure to cold for 3 hours and sacrificed immediately after cessation of stress. Note the pattern of ketosteroid distribution similar to that presented in the adrenal of a normal animal.

suggesting a reciprocal relationship between the two lipoids, at least in this area.

The reticularis contains non-carbonyl lipoid exclusively.

Exposure to cold for 72 hours. In most animals the entire cortex is stained a deep blue, most intensely in the outer zone of the fasciculata (Figure 10). By the combined stain the amount of non-carbonyl lipoid is much less than in the 24 hour specimens, and is found chiefly in the glomerulosa and only slightly in scattered cells of the other layers.

In a few animals, however, the sections show only a small amount of ketosteroid in the periphery of the fasciculata, but the amount of non-carbonyl lipoid is also considerably reduced in all layers.

Exposure to cold for 3 hours, but sacrificed 6 hours after cessation of stress. In this group the findings are as follows: Instead of the considerable depletion of ketosteroid displayed in animals sacrificed immediately after such exposure, there is an approximately five to six-fold increase in the amount of ketosteroid, as determined by the width of the ketosteroid-containing area in the fasciculata (Figure 11), and a corresponding decrease in non-carbonyl lipoid. Ketosteroid is not present in the reticularis and very little in the glomerulosa.

Single injection of ACTH immediately before exposure to cold for 6 hours

Sections from animals exposed to cold for 6 hours immediately after one intraperitoneal injection of 1.5 mgm. of ACTH do not show the depletion of ketosteroid observed in animals not receiving ACTH. The pattern of ketosteroid distribution, if not the amount of ketosteroid, is close to normal. The entire cortex shows a marked decrease in non-carbonyl lipoid and a few patches of hemorrhage (Figure 12).

Whole body x-ray irradiation (200 and 400 r)

The response to this type of stress is the same as that to cold. In animals sacrificed immediately afterward (Figure 13) the ketosteroid is greatly depleted and found only in a narrow band at the outer border of the fasciculata, and in isolated cells of the glomerulosa. This distribution persists in animals sacrificed 6 hours afterward (Figure 14), but in those sacrificed 24 hours afterward the ketosteroid is reconstituted and is found diffusely distributed throughout all the cortical layers (Figure 15).

Non-carbonyl lipoid is found in quantity in the glomerulosa and much less in the other layers. Again the reciprocal quantitative relationship of ketosteroid to non-carbonyl lipoid is observed.*

There is no difference in reaction between animals exposed to irradiation of 200 r and 400 r.

Exposure to prolonged starvation for 3 and 7 days

The ketosteroid content in animals exposed to 3 days and to 7 days of starvation is the same, but differs from that seen in other types of stress.

Three types of distribution are observed. In one type (6 animals) there is no difference from the "normal." In a second type (10 animals) the keto-

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* In 3 out of 10 animals the non-carbonyl lipoid is so markedly reduced as to suggest some direct effect on the adrenal from the radiation.

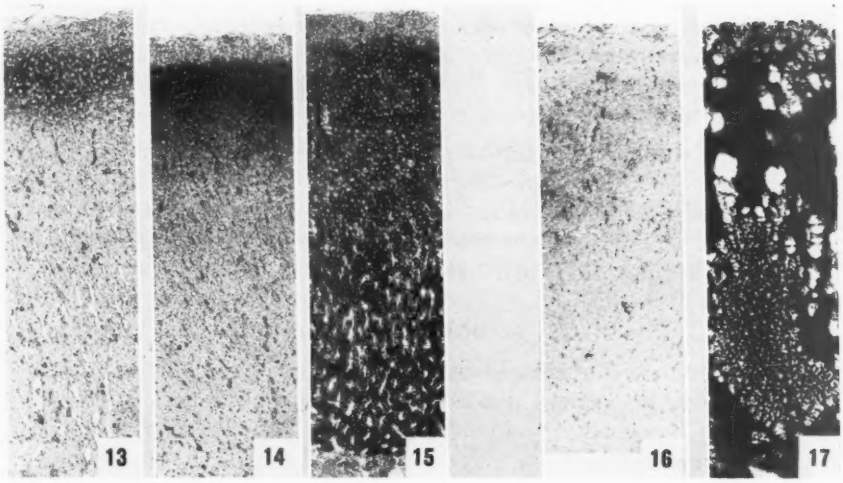

PLATE III. Cortices of female rats exposed to x-ray irradiation and prolonged starvation.

FIG. 13. Ketosteroid distribution in the adrenal of animal exposed to 400 r and sacrificed immediately (5 minutes) thereafter. Note intense depletion of ketosteroid.

FIG. 14. Progressive reaccumulation of ketosteroid in the adrenal of an animal sacrificed 6 hours after exposure to 400 r.

FIG. 15. The distribution of ketosteroid in the adrenal of an animal exposed to 200 r and sacrificed 24 hours after cessation of stress. Note the normal pattern of distribution.

FIG. 16. The adrenal of an animal exposed to starvation for a period of 7 days. Note the almost complete depletion of ketosteroid.

FIG. 17. Adrenal of an animal exposed to 7 days starvation showing "lumina" formation (see text).

steroid in the fasciculata is the same as in the "normal," but is completely absent in the reticularis. A small amount is noted in the glomerulosa. With the combined staining method areas devoid of ketosteroid are occupied by non-carbonyl lipoid. In the third type (4 animals) the ketosteroid is absent from the fasciculata and reticularis except for a small amount in isolated clusters in the mid-fasciculata. There is also a complete absence of non-carbonyl lipoid in these areas (Figure 16). The only non-carbonyl lipoid present is found in the glomerulosa. Hence in the majority, i.e. types two and three, there is failure to restore the ketosteroid deficit, which is the usual response in other forms of prolonged stress. This may be due to lack of nutritional reserves resulting from the starvation.

One animal showed "lumina" formation (Selye, H, 1950), i.e. widespread cytolysis with formation of numerous spaces containing cellular debris (Figure 17). The fasciculata and reticularis were intensely stained for ketosteroid, and, as usual, with a corresponding sparsity of non-carbonyl

lipoid, except in the glomerulosa. This condition is said to result from transitory overstimulation.

Irreversible hemorrhagic shock

The distribution of ketosteroid in the adrenals of animals in irreversible hemorrhagic shock does not differ from the "normal." This is the only type of stress used which did not induce variations in the distribution of ketosteroids. The only constant deviation from the normal was extensive hemorrhage in the adrenal parenchyma.

DISCUSSION

The depletion of ketosteroid in animals exposed to cold for 3 and 6 hours, to whole body x-ray irradiation, or to intraperitoneal ACTH suggests that in exposures to acute stress of short duration there is a rapid discharge of ketosteroid from the fasciculata and reticularis. No significant change occurs in the glomerulosa. Since the outer area of the fasciculata retains a variable amount of ketosteroid in all types of stress (with the exception of a few of the animals exposed to prolonged starvation), its persistence in this area suggests that this may be the site of elaboration or preferential deposition of ketosteroid.

After acute stress of short duration there is gradual reaccumulation of ketosteroid in the adrenal cortex. Return to the original concentration and distribution can occur within several hours following cessation of stress or during continued exposure to ACTH and stress. However, in prolonged starvation most of the animals fail to show a return to "normal," perhaps because of depletion of nutritional reserves.

It is difficult to relate the amount and distribution of ketosteroid in the adrenal to the secretory status of the gland. If the amount of ketosteroid constitutes a suitable index of the secretory activity of the gland, a return to "normal" ketosteroid pattern might be regarded as an expression of the development of resistance to stress. Conversely, the lack of such a return might indicate failure to achieve such a state.

The capacity to respond to stress is mimicked by the response to the administration of ACTH. Since depletion of ketosteroid following a single intraperitoneal injection of ACTH does not persist following repeated daily injections, it appears that repeated exposure to this stimulus produces an accelerated reconstitution of the ketosteroid to "normal." The alternative explanation of failure to respond to continued stress seems unlikely. In this connection it is noteworthy that one dose of ACTH prior to inducing acute stress is not followed by the expected depletion of ketosteroid.

A process similar to that caused by repeated exposure to ACTH might

explain the failure to observe severe depletion in most animals after prolonged starvation. In a few animals, however, both non-carbonyl containing lipoid and ketosteroid were markedly depressed, perhaps because of a deficient nutritive state. A similar observation in a few irradiated animals may be due to direct cellular injury by the radiant energy.

The absence of the normal response to stress in animals subjected to irreversible hemorrhagic shock is not easily explained. Perhaps the abrupt and persistent ischemia of the gland immobilizes the metabolic processes in the cortex in the phase of *status quo ante*. The reciprocal relationship between carbonyl and non-carbonyl lipoid suggests a ready enzymatic conversion of non-carbonyl lipoid to ketosteroid.

SUMMARY AND CONCLUSIONS

A study of the response of the adrenal cortex of adult female rats to various types of stress was made with a previously described specific method for the histologic demonstration of the active carbonyl group of ketosteroids. With the addition of Sudan IV it was possible to stain ketosteroid and non-carbonyl containing lipoid in the same section.

The types of stress used were cold, x-ray irradiation, prolonged starvation, intraperitoneal ACTH given alone, or just prior to stress, and irreversible hemorrhagic shock.

The usual response of the adrenal cortex to all these types of stress, excepting hemorrhagic shock, is an initial depletion of ketosteroid, followed by its reaccumulation with return to or toward an approximately "normal" pattern. The reaccumulation was only partial in most animals in prolonged starvation.

The return toward "normal" is evident within six hours following cessation of acute stress of short duration. If the stress is prolonged, this return occurs in most animals during the exposure.

The depletion and repletion of ketosteroid is most obvious in the fasciculata, much less so in the reticularis. Characteristic changes in the glomerulosa in response to stress cannot be demonstrated.

Shifts in ketosteroid content bear an inverse relationship to those in non-carbonyl lipoid.

Intraperitoneal ACTH reproduces the histochemical pattern of acute stress.

The "normal" pattern of ketosteroid and non-carbonyl lipoid is not changed by the induction of irreversible hemorrhagic shock in the adult female rat.

I am indebted to Drs. Jacob Fine and Arnold M. Seligman for their advice and guidance in the execution of this study.

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COMPARISON OF WATER AND SALINE HYDRATION ON DIURETIC ACTION OF SYMPATHOMIMETIC AGENTS*

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THE relationship of the adrenal medulla to water diuresis was first investigated by Stein and Wertheimer (1944). They reported that adrenal-medullectomy inhibited diuresis in water hydrated animals. The injection of l-epinephrine returned the diuresis to normal and was also noted to increase chloride excretion. Similar diuretic and chloruretic effects of epinephrine were obtained in normal animals. This action of epinephrine in normal animals was confirmed, but adrenal medullectomy was reported to be without effect on diuresis (Gaunt, Liling and Cordsen, 1945). However, it is known that epinephrine will increase the diuresis in untreated adrenalectomized rats and in adrenalectomized rats maintained on desoxycorticosterone (Hays and Mathieson, 1945; Gaunt *et al.*, 1945).

Recent studies have shown that nor-epinephrine is present in the adrenal gland and in commercial preparations of epinephrine (Tuller, 1949; Goldenberg, *et al.*, 1949; Auerbach and Angell, 1949). Because of the many differences in action of epinephrine and nor-epinephrine it is of interest to compare their diuretic properties. In the present study the diuretic effects of nor-epinephrine, epinephrine, ephedrine and privine were compared in normal rats hydrated with water. These same amines were studied during hydration with 0.85% NaCl, which normally produces a delayed urine excretion. Observations were also made on the chloruretic effect of these substances.

METHODS

The diuresis studies were performed on normal male albino rats weighing between 200 and 300 grams and fed a commercial stock diet. The rats were hydrated according to the method of Ham and Landis (1942) in which two doses of water are administered. The first dose of water, 2.5% of body weight, is given and two hours later, a second dose of 5% of body weight is administered. Water *ad libitum* was allowed until the time of the first hydration. The amines to be studied were injected subcutaneously in water solution 15 to 20 minutes before the second dose of water. Each test was performed on groups of three animals. After the second hydration urine measurements were made every 15 minutes for three hours. The results are plotted in a standardized manner and the area under each curve is measured in square inches with a planimeter (Ham and

Received for publication June 18, 1951.

* This study was aided by a grant from Parke, Davis and Company.

Landis, 1942). The resultant area is recorded in square inches in the tables. In studies with normal saline, water was given as the first hydration and 0.85% saline as the second hydration. The amines injected were l-epinephrine HCl (containing less than 0.1% nor-epinephrine), l-nor-epinephrine bitartrate monohydrate, ephedrine sulfate and privine hydrochloride.

RESULTS

Control animals: A typical excretion curve obtained in control animals hydrated with water or 0.85% NaCl is shown in Figures 1 and 2. Indi-

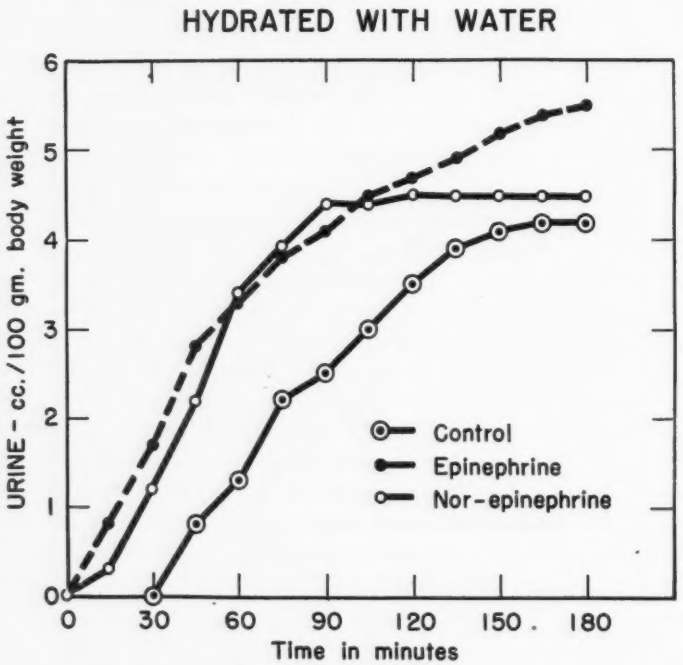

FIG. 1. Effect of l-epinephrine and l-nor-epinephrine on urine excretion of water-hydrated animals.

vidual data, tabulated in Tables 1 and 2, showed that such results are quite constant and easily reproduced. Hydration with 0.85% always produces a low urine output when compared with water hydrated animals, the average difference in two control studies being 9.06 sq. in. in table 1 and 10.24 sq. in. in table 2.

Epinephrine and nor-epinephrine: Epinephrine and nor-epinephrine in doses of 100 μg. per 100 gm. of body weight produced a marked diuretic effect in animals hydrated with water or 0.85% NaCl. The epinephrine

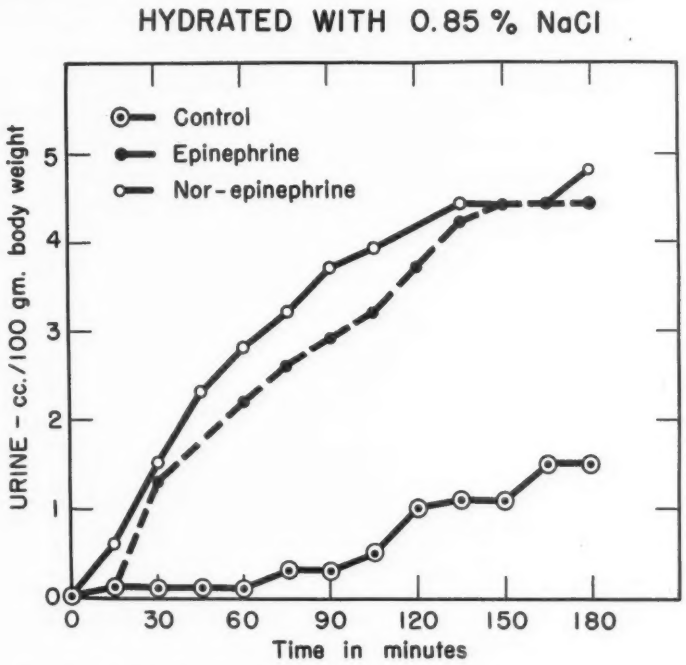

FIG. 2. Effect of l-epinephrine and l-nor-epinephrine on urine excretion of rats hydrated with 0.85% NaCl.

TABLE 1. DIURETIC EFFECTS OF EPINEPHRINE AND NOR-EPINEPHRINE IN RATS. DATA INDICATES DIFFERENCE FROM CONTROLS AS MEASURED IN SQUARE INCHES

	Both Hydrations H_2O			2nd Hydration 0.85% NaCl		
	Control	Epinephrine 100 μg./ 100 gm.	Nor-Epinephrine 100 μg./ 100 gm.	Control	Epinephrine 100 μg./ 100 gm.	Nor-Epinephrine 100 μg./ 100 gm.
	12.87	+13.86	+13.04	2.63	+18.31	+19.14
	15.60	—	+11.62	6.09	—	+12.66
	18.18	+8.70	+10.77	9.27	+13.47	+10.44
	13.65	+11.61	—	8.49	+13.10	—
	13.11	+11.97	+5.37	4.32	+8.17	+6.60
	16.62	+1.80	—	5.82	+13.51	—
	17.16	—	+8.54	7.08	+9.03	+15.65
	16.81	—	+2.57	8.04	—	+16.95
	13.80	+8.17	+6.36	5.94	+10.35	+12.87
	13.80	+7.26	—	3.36	—	+13.17
Av.	15.16	+9.05	+8.32	6.10	+12.28	+13.44
S.E.	±.61	±1.50	±1.46	±.69	±1.3	±1.37

produced an average increase of 9.05 sq. in. in the area or curve following water hydration, comparable to that obtained with nor-epinephrine (Table 1). The animals receiving the amines also show a prompt onset of diuresis following the second hydration (Fig. 1). The time required to excrete 50% of the hydration may also be estimated from the curve. Fifty per cent excretion is represented by 2.5 cc. urine per 100 gm. of body weight. The time required for 50% excretion in the control animals illu-

TABLE 2. DIURETIC EFFECTS OF EPHEDRINE AND PRIVINE IN RATS. DATA INDICATES DIFFERENCE FROM CONTROLS AS MEASURED IN SQUARE INCHES

	Both Hydrations H_2O			2nd Hydration 0.85% NaCl		
	Control	Ephedrine 100 μg./ 100 gm.	Privine 25 μg./ 100 gm.	Control	Ephedrine 100 μg./ 100 gm.	Privine 25 μg./ 100 gm.
	15.18	+2.43	+6.78	4.02		+12.09
	18.27		+7.59	5.49	+0.27	+21.54
	13.38	+3.88	+10.41	3.18	+2.48	+4.50
	15.45	−3.45	+9.66	4.14	−3.00	+14.58
	15.75	+0.47	+0.64	4.95	−0.24	+3.60
		+0.07	+5.70		+1.59	+13.44
	14.64	+5.88	+2.07	5.40	+1.37	+4.86
	11.85	−2.82	+2.25	6.72	+0.54	+9.72
	15.51	+1.23	+7.14	4.16	−1.19	+7.57
Av.	15.00	+0.96	+5.80	4.76	+0.23	+10.21
S.E.	±0.66	±1.11	±1.07	±0.39	±0.61	±1.95

strated in Figure 1 is 88 minutes, after epinephrine 47 minutes, and after nor-epinephrine 38 minutes. Following hydration with 0.85% NaCl both amines produced a marked increase in urine flow and total volume, returning the rate of excretion to the level obtained in animals hydrated with water (Fig. 2, Table 1).

Ephedrine and privine: Ephedrine sulphate was without diuretic effect in animals hydrated with water or with animals who received the second hydration as 0.85% saline. The privine administered had a diuretic action irrespective of the hydrating solution (Table 2), although the effect of privine was slightly less than that obtained with epinephrine or nor-epinephrine.

Chloruretic effect of the amines: Epinephrine and nor-epinephrine showed a definite chloruretic effect in animals that were hydrated with water. This was true when the results were calculated on the basis of chloride per cc. of urine, total chlorides, or total chlorides per 100 grams of body weight (Table 3). The increase in chloride excretion was much greater in proportion to the increase in urine volume produced by these amines. When the hydrating solution was 0.85% sodium chloride, the epinephrine and nor-epinephrine did not show a chloruretic effect. In these instances the urine

TABLE 3. CHLORURETIC EFFECT OF EPINEPHRINE AND NOR-EPINEPHRINE. RESULTS IN MICRO-EQUIVALENTS OF CHLORIDE

	Cl/cc. urine			Total urine Cl			Total urine Cl/100 gms. Body weight		
	Control	Epin.	Nor-Epin.	Control	Epin.	Nor-Epin.	Control	Epin.	Nor-Epin.
				Both Hydrations H_2O					
	4.00		13.65	104.80		529.62	16.10		79.60
	3.50	31.00	12.53	91.00	1023.00	438.55	17.70	193.00	77.90
	5.50	102.00	25.00	170.50	4800.00	975.00	25.90	782.50	168.50
	6.50	42.50		182.00	1920.00		27.65	281.00	
	1.99	35.00	41.50	49.75	476.00	1162.00	6.38	63.40	157.00
	7.49	43.50		246.00	1619.00		28.70	211.50	
	6.50		27.50	162.50		1023.00	29.10		160.50
	8.49		18.03	278.50		505.00	41.65		77.70
	6.50	60.00	27.50	233.60	2916.00	951.50	28.30	334.00	123.30
		40.00			1960.00			230.60	
Av.	5.61	50.57	23.67	168.74	2102.00	797.81	24.61	299.43	120.64
S.E.	±0.691	±9.59	±3.79	±25.48	±535.7	±112.2	±3.42	±85.9	±15.9
				1st Hydration H_2O, 2nd Hydration 0.85% NaCl					
	85.75	93.00	95.00	805.05	3069.00	2660.00	135.20	575.00	460.00
	87.00		73.75	365.40		2197.75	57.80		350.50
	140.00	129.50	137.50	2380.00	5440.00	2670.00	388.00	840.00	593.00
	112.50	121.00		2025.00	5325.00		322.00	794.00	
	141.00	108.50	110.50	2055.00	2755.00	3625.00	248.00	327.50	408.00
	122.75	117.00		1766.00	3930.00		205.50	580.00	
	113.00	133.00	88.00	1650.00	3375.00	2905.00	256.50	487.00	454.00
	112.00		104.00	1613.00		3410.00	259.00		566.00
	140.25	124.00	108.00	1879.35	4761.66	3974.40	235.20	582.10	474.30
	176.00	118.00		2076.80	4956.00		245.80	565.10	
		131.50			4812.90			615.40	
Av.	123.03	119.50	102.39	1661.66	4269.39	3063.16	235.30	596.23	472.26
S.E.	±8.64	±4.21	±7.58	±187.34	±335.00	±237.00	±91.00	±50.7	±31.9

TABLE 4. EFFECT OF EPHEDRINE AND PRIVINE ON CHLORIDE EXCRETION. RESULTS IN MICRO-EQUIVALENTS OF CHLORIDE

	Cl/cc. urine			Total urine Cl			Total urine Cl/100 gms. Body weight		
	Control	Ephedrine	Privine	Control	Ephedrine	Privine	Control	Ephedrine	Privine
				Both Hydrations H_2O					
	7.48	7.02	41.75	190.12	210.75	1444.55	31.2	35.4	266.2
	20.45		33.00	584.87		1221.00	95.9		191.1
	5.50	8.49	35.10	174.90	263.04	1593.54	23.1	33.6	195.0
	7.48	3.51	40.50	284.43	100.39	1773.90	32.9	13.6	235.5
	4.39	2.05	12.00	147.50	56.60	300.00	19.5	8.3	49.8
		4.39	16.10		143.70	735.00		20.5	91.7
	3.51	2.98	1.50	108.20	91.20	39.80	16.8	14.4	6.8
	5.75	2.50	9.50	186.20	55.00	309.00	22.7	7.7	39.2
	4.50	2.00	34.00	157.50	80.80	1321.00	18.4	10.1	190.0
Av.	7.38	4.12	24.83	229.22	125.18	907.87	32.6	18.0	140.6
S.E.	±1.87	±0.85	±4.84	±5.37	±26.50	±196.5	±9.27	±3.89	±31.20
				1st Hydration H_2O, 2nd Hydration 0.85% NaCl					
	106.5	67.0	118.0	1278.0	603.0	4720.0	190.5	37.0	689.1
	132.0		115.0	1478.4		3312.0	231.0		556.0
	163.5	130.5	115.0	1569.6	1774.8	1472.0	198.7	217.5	212.4
	148.5	95.0	126.0	1336.5	266.0	4410.0	176.8	35.4	543.7
	30.5	80.8	95.0	304.7	807.5	1273.0	44.0	114.3	203.0
			117.0			3930.0			567.5
	59.0	92.5	124.0	531.0	1210.0	1687.0	78.3	166.7	281.5
	118.0	120.0	101.3	1720.0	1730.0	3240.0	231.0	207.0	417.0
	89.0	129.5	160.0	928.0	830.0	3900.0	114.2	115.0	468.0
Av.	105.9	102.2	119.0	1143.3	1031.6	3105.0	158.1	136.1	437.6
S.E.	±15.91	±9.38	±6.12	±179.44	±237.70	±437.0	±24.30	±24.60	±63.90

chlorides per cc. are the same. The total chloride excretion is increased but no greater than that accounted for by the increase in urine volume produced by these amines. Privine showed a chloruretic effect approximating that of nor-epinephrine (Table 4). This effect was obtained only in animals hydrated with water and not when the hydration was with 0.85% saline. Ephedrine was without chloruretic effect in the water hydrated or saline hydrated animals.

DISCUSSION

The subcutaneous injection of 1-epinephrine 100 μg/100 gm. of body weight produced a diuresis and chloruresis in animals hydrated with water, confirming the earlier studies of Stein and Wertheimer (1944) and Gaunt *et al.* (1945). It is interesting that Silvette (1942), in studying the decreased urine volume of hydrated animals during sodium thiopental anesthesia, noted that epinephrine would return the rate of excretion to normal. In the present studies the administration of 1-nor-epinephrine produced a prompt and sustained diuresis, comparable to that obtained with epinephrine (Fig. 1). The nor-epinephrine was also markedly chloruretic; increasing both the concentration of chloride per cc. of urine and the total amount per 100 grams of rat body weight.

Recently Horres and associates (1950) have reported on the diuretic action of epinephrine and nor-epinephrine in water hydrated animals. They noted that only nor-epinephrine produced a diuretic effect while epinephrine administered in doses of 3 μg. or 30 μg. per 100 gm. of body weight failed to produce this response. With larger doses, as used in our studies, 1-epinephrine gave both a diuretic and chloruretic effect.

Hydration with normal (0.85%) saline produces only a slow urine excretion of low total volume (Fig. 2). Both epinephrine and nor-epinephrine were found to exert a marked diuretic effect, even under these conditions, returning the excretion rate and volume to the level obtained in water-hydrated animals. Hydration with 0.85% NaCl should serve as a useful screening procedure to detect amines with diuretic effects. Neither epinephrine nor nor-epinephrine increased the concentration of chloride in the urine. Although the total excretion of chloride was increased, the change was not greater than that accounted for by the increase in urine volume.

Stein and Wertheimer (1942) reported that ephedrine was without diuretic effect in water-hydrated animals. In our own studies, with water or saline hydrations, a similar lack of effect was noted. Chloride excretion was also unchanged. Privine, on the other hand, increased urine excretion irrespective of the type of hydration, but a chloruretic effect was obtained only in water-hydrated animals.

The possible role that the adrenal medulla may play in the response to

water or saline tolerance tests is not clear. Studies of the effect of adrenal-medullectomy on diuresis have produced conflicting results (Stein and Wertheimer, 1944; Gaunt *et al.*, 1945). The low urine output of adrenalectomized animals is definitely increased by epinephrine (Stein and Wertheimer, 1944; Hays and Mathieson, 1945; Gaunt *et al.*, 1945). It is also known that adrenalectomized rats maintained on adrenal extract or desoxycorticosterone (DCA) lose their ability to excrete water. However, the excretion of the DCA maintained rat, particularly studied by Hays and Mathieson, can be returned to normal by the injection of epinephrine. This effect of epinephrine in the otherwise refractory animal is of interest in evaluating the role of adrenal medulla in urine secretion.

SUMMARY

The administration of l-epinephrine, l-nor-epinephrine or privine produces diuresis in normal rats hydrated with water. Chloride excretion was also increased in these animals.

Saline hydration markedly delays urine excretion. Under such conditions l-epinephrine, l-nor-epinephrine and privine exerted a marked diuretic action, but were without chloruretic effect.

With the doses used the effects of epinephrine and nor-epinephrine were similar. Ephedrine, irrespective of the hydrating solution, was without diuretic or chloruretic effects.

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FRUCTOSE AND VOLUME RELATIONSHIPS OF BOVINE SEMEN

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SEVERAL investigators have reported reducing sugar as a constituent of mammalian semen according to Mann (1951). Yamada (1933) was probably the first to indicate the occurrence of fructose in semen and his information was based on a qualitative test made on human material. Mann (1949) has reviewed the literature relative to the isolation and identification of the seminal sugar as D(−) fructose. Mann (1951) stated that the following species have been examined and found to contain fructose in the semen: man, bull, ram, rabbit, boar, stallion, goat, guinea pig, rat, mouse, hamster, and opossum. The fructose concentration varies among species and it ranges from a few milligrams per cent in the boar and stallion to several hundred in the rabbit, ram, bull, and man (Mann, 1949).

Huggins and Johnson (1933) were the first to show that the high concentration of reducing sugars in human semen is due principally to the secretion of the seminal vesicles and not the prostate. Similar observations have been made by Bernstein on the bull (1937), McKenzie, Miller and Bauguess on the boar (1938), and Moore and Mayer (1941) on the ram.

A comparative study of fructose distribution in various accessory organs has demonstrated that in many species fructose originates chiefly in the seminal vesicles. However, in the bull and ram some fructose was found in the ampulla. According to Mann (1949) the fructose level in fresh semen depends upon the secretory activity of the accessory glands, particularly the seminal vesicles; and the functional status of these glands is largely dependent upon the male sex hormone level. A low fructose content of semen would indicate a decreased secretory activity of the accessory glands whereas a high level would suggest a greater physiological activity of these organs.

According to Mann (1949) experiments on rabbits and bull calves have clearly demonstrated that in young animals fructose appears in the acces-

[Received for publication June 27, 1951.

* This project is supported in part by funds furnished by Dairy Genetics, Des Moines, Iowa; Eastern Iowa Artificial Breeding Association Cedar Rapids, Iowa; and Northwest Iowa Federated Breeders Coöp., Sheldon, Iowa. Project *936*.

Journal Paper No. *1966* of the Iowa Agricultural Experiment Station, Ames, Iowa.

sory glands at any early stage when the testes are still incapable of producing viable spermatozoa. Inasmuch as the formation of seminal fructose is dependent upon the presence of male sex hormone, it has been suggested that this hormone is of important physiological significance before the onset of spermatogenesis. As Mann and Parsons (1947) have indicated, one of the essential functions of testicular hormone is its role in the metabolic transformation responsible for the formation of the nutrient material for spermatozoa in the form of fructose.

A study of the factors controlling the composition and volume of semen is important in the physiology of reproduction and this is particularly true as related to artificial insemination. The investigation reported here deals with the relationship between fructose content and the volume of semen produced by bulls of several breeds.

METHODS

Bulls of seven breeds of cattle, Holstein, Guernsey, Ayrshire, Brown Swiss, Jersey, Shorthorn, and Angus, were used in this study. Collections of semen were made by experienced technicians each weekday between 7 and 8 a.m. during the last three weeks of September and the first week of October, 1950. A collection was made from each bull once per six-day interval and the data presented were obtained from the first ejaculation. The volume of the ejaculate was determined by collection in a graduated centrifuge tube and the total number of sperm was estimated by counting in a hemocytometer. Fructose content of semen was determined according to the method of Roe (1934). For measurement of color density, a Klett-Summerson photoelectric colorimeter was used with a green filter (No. 54). Analysis of variance was used in the statistical treatment of the data (Snedecor, 1946).

RESULTS

Data obtained from this study are presented in Table 1.

TABLE 1. SUMMARY OF BREEDS, NUMBER AND AGE OF BULLS, VOLUME, SPERM COUNT, FRUCTOSE CONCENTRATION, AND FRUCTOSE CONTENT OF SEMEN

Breed	No. of bulls	Average age in years	Total no. of samples	Average volume of ejaculate cc.	S.D.*	Average sperm count, millions per cmm.	S.D.	Average fructose concentration mg./cc.	S.D.	Average fructose per ejaculate mg.	S.D.
Holstein	9	6.0	23	6.2	2.3	1.726	0.5	5.7	1.9	37.1	22.4
Guernsey	6	7.0	24	7.1	2.8	1.525	0.4	6.1	1.6	45.1	23.9
Ayrshire	3	2.9	10	5.0	1.3	1.633	0.7	5.0	1.4	23.7	12.4
Brown Swiss	4	9.2	16	5.9	1.3	1.546	0.4	4.8	1.1	28.9	9.4
Jersey	4	7.0	16	4.2	1.2	1.386	0.5	5.5	1.5	23.5	9.6
Shorthorn	7	7.0	26	4.7	1.7	1.639	0.5	3.5	1.2	17.0	7.3
Angus	3	1.7	11	3.5	1.8	1.520	0.5	5.0	1.1	17.2	4.8

* S.D.—Standard Deviation.

DISCUSSION

The data presented in Table 1 indicate there was a significant breed difference in the volume of the ejaculate. It is possible to place the breeds studied into three groups with respect to volume of ejaculate. The lowest

volume was produced by the Angus; the Jersey, Shorthorn, and Ayrshire were intermediate; and the Brown Swiss, Holstein, and Guernsey produced relatively larger quantities. The sperm density was quite uniform for these seven breeds as indicated by these data. Furthermore, these results indicate that there was no constant relationship between sperm density and fructose concentration of the semen. This confirms the earlier findings of Mann (1948) based on an exhaustion test. In this particular experiment a bull produced eight ejaculations in 63 minutes with a marked decrease in sperm density. However, this fall was not accompanied by a corresponding decrease in fructose concentration of the semen.

In evaluation of the data summarized in Table 1, considerable variation was found in both the concentration and in the total fructose content of the ejaculate. The concentration varied from 60 to 1070 mg. per 100 cc. of semen for a Shorthorn and Holstein, respectively, with an average for all breeds of 510 mg. per 100 cc. The highest concentration was in an ejaculate of 9 cc. with a total fructose content of 96 mg. The total fructose ranged from 0.9 to 101 mg. per ejaculate for a Shorthorn and Guernsey, respectively, with a mean of 28 mg. for all animals. The ejaculate with the highest fructose content in the series contained 101 mg. of fructose in 15 cc. of semen.

Statistical analysis of the data summarized in Table 1 indicated a significant difference ($P = 0.01$) among the 9 Holstein bulls studied with respect to volume of ejaculate, the concentration of fructose, and the total fructose content of the ejaculate but the regression coefficient of fructose concentration on the volume of ejaculate was not significant for these animals. In the case of the data for the Guernsey breed there was a significant difference ($P = 0.01$) among the 6 bulls studied with respect to the volume of ejaculate, concentration of fructose, and total fructose output. Furthermore, the regression coefficient of the fructose concentration on the volume of the ejaculate was significant at $P = 0.01$ among these animals. This relationship is presented graphically in Fig. 1.

Analysis of the data on the volume of the ejaculate of the Ayrshire, Brown Swiss, Jersey, Shorthorn, and Angus bulls did not indicate a significant difference among the individual animals of each of these breeds. The fructose concentration of the semen of the Ayrshire, Shorthorn, and Angus bulls did not differ significantly among the individuals of each breed studied, however, in the case of the Brown Swiss and Jersey there was a significant difference ($P = 0.01$). The regression coefficient of the fructose concentration on the volume of the ejaculate of the individual animals of each of these 5 breeds, was not significant. Of these, the Jersey and Angus bulls showed a significant difference among the animals with respect to total fructose output in the ejaculate ($P = 0.05$).

The variations in volume and fructose content observed in the different breeds and individuals may result from marked changes in the size and functional status of the seminal vesicles. These may be related to periodic physiological changes associated with the level of male sex hormone as well as the reactivity of the secreting tissue. The results of this investigation,

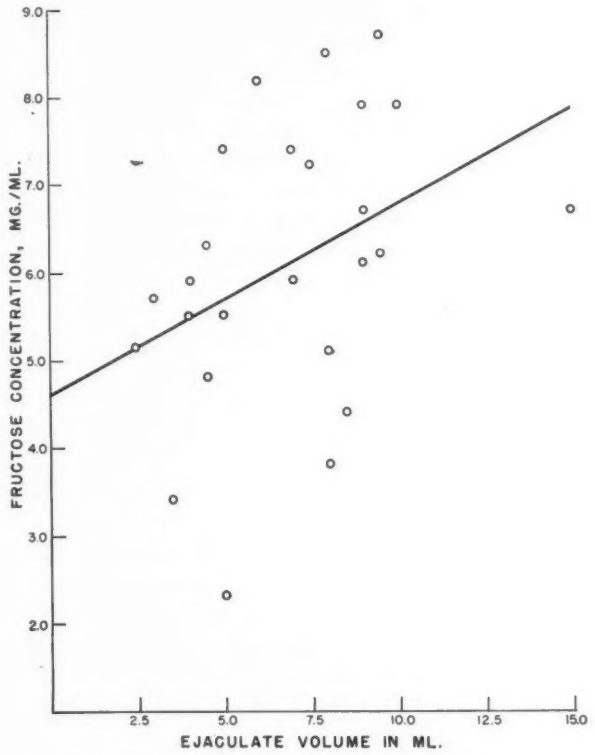

FIG. 1. Relation between fructose concentration and ejaculate volume for Guernsey bulls. ($Y = 0.2197x + 4.594$)

as presented in Table 1, indicate that the total fructose produced by the bovine is directly correlated with the secretory activity of the accessory glands.

In a study of the relation between the volume and fructose content of human semen, Harvey (1948) found considerable variation in the concentration of fructose and in the total fructose content of the ejaculate. The concentration varied from less than 5 to 640 mg. per 100 cc. with a mean

of 287 mg. per 100 cc., while the total fructose ranged from less than 0.05 to 31.6 mg. with a mean of 9.37 mg. per ejaculate.

SUMMARY

The average ejaculate volume, sperm count, and fructose concentration were determined on 36 bulls representing 7 breeds of cattle: Holstein, Guernsey, Ayrshire, Brown Swiss, Jersey, Shorthorn, and Angus. The smallest average ejaculate volume, 3.5 cc., was produced by the Angus and the largest by the Guernsey, 7.1 cc. The average sperm count was fairly uniform for all breeds. The average fructose concentration was lowest for the Shorthorn at 3.5 mg./cc. and highest for the Guernsey at 6.1 mg./cc. The regression coefficient of the fructose concentration on the volume of the ejaculate was significant at $P = 0.01$ for the Guernsey bulls studied. The Guernsey bulls produced an average of 45.1 mg. of fructose per ejaculate whereas those of the Shorthorn and Angus breeds produced 17.0 and 17.2 mg., respectively.

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THE EFFECTS OF CORTISONE *IN VIVO* ON THE OXIDATIVE METABOLISM OF THE RAT ADRENAL

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IT HAS been demonstrated that repeated injection of large doses of cortisone acetate into rats results in, among other effects, atrophy of the adrenal cortex which is restricted practically entirely to the zonae fasciculari and reticularis (Ingle, Higgins and Kendall 1938; Wells and Kendall 1940). Decrease in the *number* of cells makes only a minor contribution to the reduction in size of the cortex, this being due primarily to a decrease in the *size* of the cells in the two affected layers which show virtually complete loss of lipid (Winter, Silber and Stoerk 1950). One would not expect loss of lipids, *per se*, to be reflected in any reduction in the capacity of the adrenals of treated animals to oxidize substances such as the intermediates of the Krebs' cycle since these lipids are presumably not part of the cellular enzymatic apparatus. Of course, the lipids may serve normally as substrates in the intact gland so that their disappearance from the tissue may be reflected in a decreased endogenous respiration of adrenal preparations.

It was considered of interest to go beyond these morphological studies and to determine whether the cortisone-induced atrophy is paralleled by any quantitative changes in the enzyme activity of the rat adrenal. In this work we have concerned ourselves with a number of the oxidations occurring in the tricarboxylic acid cycle, and we have found that the rat adrenal, like other tissues of this animal, oxidizes the intermediates of the Krebs' cycle. Furthermore, the rates of various oxidations in this cycle are substantially reduced in the "cortisone-atrophic" adrenals.

EXPERIMENTAL

A series of experiments was performed in which male albino rats weighing about 175 grams were used. In each experiment the animals were divided into two groups. (a) Each rat in the "treated" group received a daily subcutaneous injection of 3 mg. of cortisone suspended in 0.6 ml. of saline for 6 days. (b) Rats in the control group were similarly injected, receiving 0.6 ml. of saline in each injection. Twenty-four hours after the last injections the rats were killed and the adrenals removed, cleared of as much adhering connective tissue as possible, weighed and finally homogenized in an appropriate volume of isotonic KCl, using a glass homogenizer.

A standard reaction mixture was used, made up of the following ingredients (final concentrations given): K phosphate buffer, pH 7.4 (0.007 M); KCl (0.115 M); K adeny-

Received for publication June 27, 1951.

late (0.001 M); $MgCl_2$ (0.004 M); substrate neutralized to pH 7.4 with KOH (0.016 M). 0.2 ml. of the homogenate containing about 30 mg. wet weight of tissue was added to give a total volume of 1.0 ml. Standard manometric techniques were employed for the measurement of oxygen uptake (Umbreit *et al.* 1949). All incubations were performed at 37°.

RESULTS

Two methods were used for the statement of results. In one, the oxygen uptake per unit weight (Q_{O_2}) of adrenal tissue is given (Table 1A). Since

TABLE 1. OXYGEN UPTAKE BY HOMOGENATES OF NORMAL RAT ADRENALS AND CORTISONE-INDUCED ATROPHIC RAT ADRENALS

Substrate	A		B	
	Q_{O_2}[1]		Microliters of oxygen uptake in 30 minutes per average weight of adrenals	
	Controls	Treated	Controls	Treated
None	1.1	−1.3	4	−3
Citrate	7.3	3.8	27	9
Cis-Aconitate	7.6	3.4	28	8
α-Ketoglutarate	10.3	6.8	38	16
Succinate	13.3	14.1	49	33
Fumarate	4.9	1.7	18	4
l-Malate	2.7	2.6	10	6

[1] Q_{O_2} = microliters of oxygen consumed per hour per mg. dry weight of tissue.
In the above table, the Q_{O_2} values have been calculated from the uptake in the first 30 minutes of incubation, when as: the rate was linear. Other details in the text.

whole adrenals were used and there were unequal proportions of cortex and medulla in the two sets of rats this method tends to increase the relative contribution to the total figure made by the medulla in the "treated" group.

In the alternative method the oxygen consumption data are compared "gland for gland" in the two groups (Table 1B). This expression is thus based on equal weights of medulla (which does not undergo any change in the course of cortisone treatment) and varying amounts of cortex. The reduction in weight of the adrenals of the treated group was similar to that previously observed by Winter *et al.* (1950). The average weight of a pair of control adrenals was 33.5 mg., that of "treated" adrenals was 21.3 mg. (Standard error of the mean, 1.3 mg.)

Curves showing oxygen consumption as a function of time are plotted in Figure 1. These data represent "extra oxygen consumption" due to presence of substrate, i.e. (total O_2 uptake)—(endogenous uptake). Respiration of adrenal homogenates of the cortisone-treated rats in the absence of added substrate was usually close to zero. Two points may be noted in

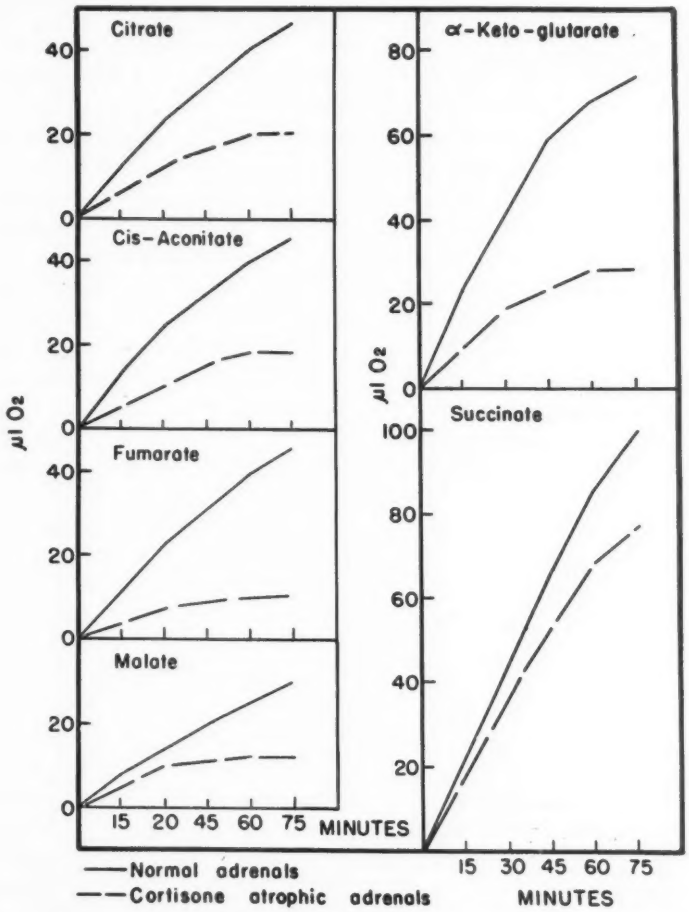

FIG. 1. Oxygen uptake by homogenates of normal and of cortisone-atrophic rat adrenal glands in presence of various substrates.

Graphs are plotted from data for "extra oxygen consumption" due to presence of substrate. Microliters of oxygen per average weight of adrenals.

Figure 1: (1) The rates of oxygen consumption of control homogenates were rather steady for about an hour, but in the case of the homogenates from cortisone treated animals these rates tended to fall off earlier. (2) The oxygen uptake by the "treated" homogenates in the earlier portion of the incubation period is about 50% or less of the uptake by control homogenates for all substrates except succinate, where the decrease is only about 20%.

Hartman and Brownell (1949) provide data indicating that the medulla of the male albino rat makes up less than 10% of the total weight of the adrenal. On this basis the observed decline in weight of the adrenals of the cortisone-treated group would represent a decrease of 35–40% in the amount of cortical tissue. If one grants that the oxidative capacity of medulla and cortex, weight for weight, are not greatly disparate, it follows that a decrease in the oxidative rate due simply to loss of cortical substance should not be expected to exceed the above figure. Actually, as seen in Figure 1 and Table 1B, the decrease is greater than this.

In studies of intermediary metabolism of adrenalectomized rats it has been observed (Umbreit and Tonhazy, 1951) that homogenates prepared from tissues of such animals require supplementation with more adenylic acid (or adenosine triphosphate) than do homogenates of normal tissues in order to bring the rates of oxidation up to the level of the controls. Without this increased supplement a picture somewhat similar to that in Figure 1 is seen: lowered O_2 uptake and earlier plateauing of the curves of substrates requiring the presence of the adenylate system for oxidation, but not of succinate which has no requirement for adenylate. However, it was not possible to implicate the adenylate system in this case, for increasing the concentration of adenylate threefold had no effect on the oxidation of alpha-ketoglutarate (or succinate) by adrenals of cortisone-treated animals.

DISCUSSION

While the marked inhibition of oxidations in the adrenals of cortisone-treated rats could not be predicted on the basis of the atrophy resulting from this treatment, nevertheless the two phenomena are consonant. It is generally assumed that the suppression by cortisone of ACTH release from the pituitary is the probable cause of the cortisone-induced atrophy of the adrenals. The decreased output of steroid hormones by the adrenal cortex as a consequence of reduced ACTH secretion is well-known (Sayers, 1950). In the light of the present observations it would then seem that the atrophy of the adrenal cortex due to reduced ACTH output from the anterior pituitary is accompanied by reduction in the activity of at least some of the enzyme systems of this tissue to a greater extent than would be expected on the basis of decreased cortical size.

It may be worthwhile mentioning parenthetically the recent attempts to demonstrate succinic dehydrogenase activity in the adrenals of various species by histochemical techniques. While Seligman and Rutenberg (1951) obtained negative results in rat and mouse with ditetrazolium dichloride, Zweifach and co-workers (1951) had no difficulty in detecting this enzyme using 2,3,5-triphenyl tetrazolium chloride. Our results, obtained by the

direct measurement of oxygen uptake in Warburg vessels, are in agreement with the latter findings.

SUMMARY

Cortisone was administered to young adult male albino rats in doses of 3 mg. per day subcutaneously for 6 days in order to induce the reversible atrophy of the adrenal cortex previously reported by others. Homogenates were prepared from such atrophied glands as well as from adrenals of normal control rats run in parallel, and oxidative metabolism was studied. Adrenals of cortisone-treated rats show a decreased endogenous respiration as well as greatly reduced rates of oxidation of various intermediates of the Krebs' cycle. These decreased rates cannot be accounted for merely by the decreased amount of cortical tissue in the atrophied glands.

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FLUID AND ELECTROLYTE SHIFTS IN THE HYDRATED ADRENALECTOMIZED RAT[1]

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IT IS a well established fact that the normal diuresis which follows the ingestion of large amounts of water is impaired in the adrenalectomized animal (references cited by Gaunt *et al.*, 1949). It has been shown in the rat that this retention of water is due for the most part to an increased sensitivity of the renal tubules to an antidiuretic substance in the blood which accumulates after adrenalectomy. Also, the adrenal cortical hormones themselves are apparently necessary for a high rate of water excretion. (Gaunt *et al.*, 1949; Birnie *et al.*, 1950).

The experiments here reported were conducted in order to determine the sites of the retained fluid in hydrated adrenalectomized rats and what role shifts in electrolytes might play in this regard. It seemed advisable to use the sodium space, as measured with radiosodium 22, rather than the thiocyanate space, as an index of extracellular fluid volume. Recent investigation has shown that a large fraction of an injected quantity of thiocyanate becomes bound to the serum albumin (Schienberg *et al.*, 1950). Since there may be an increased capillary permeability to serum proteins during adrenal insufficiency, the thiocyanate space then would not be a consistent index of the extracellular fluid volume. Furthermore, it has also been reported that due to interfering chromogens, urinary thiocyanates are difficult to determine accurately (Doxiadis and Gairdner, 1948). All fluid compartment determinations were made on both the intact and gutless rats in order to eliminate any error in the determination of sodium space which would arise if the sodium concentration in the gut were higher than the serum concentration. This also afforded a means of determining in which compartments absorbed but unexcreted water was located. Serum electrolytes and fluid compartment determinations were made on both non-hydrated and hydrated normal and adrenalectomized rats.

MATERIAL AND METHODS

Normal and two-day adrenalectomized male rats weighing between 140–160 grams were starved approximately 22 hours before use. The adrenalectomized animals received

Received for publication July 9, 1951.

[1] This investigation was supported in part by a research grant from the Atomic Energy Commission.

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no extra salt or hormone therapy. The animals were weighed, injected intraperitoneally with approximately 3 microcuries of Na^{22} as sodium chloride, and placed in metabolism cages for a period of 2 hours. In addition, the rats to be hydrated were also given two doses of water (5 cc. per 100 gm. of weight) by stomach tube, an hour apart. At the end of the two-hour period, the animals were removed, urine excretion measured, and the metabolism cages washed down with distilled water. The urine, then diluted to a known volume, was collected in order to determine the amount of radiosodium excreted. The rats were weighed again and a known quantity of blood was removed by heart puncture and centrifuged for serum electrolyte and radiosodium determinations. The animals' stomachs and intestines were removed and weighed and the carcasses were dried to a constant weight at approximately 110° C. The calculated dry weight of the removed blood was then added to the dried carcass weight. The gut was also dried to a constant weight after its radiosodium activity was determined. The radiosodium activities of the serum, urine, and gut were measured in respect to standards of the injection solution by means of a cup-type (Toroidal) gamma counter.[3] All samples were counted twice and for a sufficient period of time to give one per cent accuracy.

Serum sodium and potassium values were determined by means of a Beckman Flame Spectrophotometer according to the method described by Mosher *et al.* (1949). Serum chlorides were measured by the method of Sendroy as modified by Van Slyke and Hiller (Hawk, Oser, and Summerson, 1947).

Total body water values were obtained by dividing the water content of the intact or gutless animal by its corresponding nonhydrated weight after the two-hour run. In the hydrated animals this latter weight value was obtained by subtracting from their pre-run weight, the average weight loss during this two-hour period of the nonhydrated animals. In this way, changes in total body water could be read directly as cc. per 100 gms.

The two-hour sodium space which serves as an index of the extracellular fluid volume was determined from the following formula:

$$\frac{\text{counts/second injected} - \text{total counts/second excreted in urine}}{\text{serum activity in counts/second/cc.}} \times 100 \div \text{nonhydrated body weight at the end of the run in grams.}$$

Again changes in the sodium space could be read directly as cc./100 gms.

The extra-sodium space (the difference between the total body water and sodium space) was used as an index of intracellular space. The total sodium in the two-hour sodium space was obtained by multiplying the sodium space by the serum sodium concentration. Use of the Gibbs-Donnan equilibrium ratio was not considered necessary for this study.

Intestinal absorption was obtained by comparing the gut weights of starved hydrated rats with those which had received no water. In all cases mentioned above, control and adrenalectomized rats were run simultaneously

RESULTS

As shown in Table 1, the adrenalectomized rats retained over twice as much of the ingested water as did their controls. This difference in retention was due in part to both a sluggish gastric motility and to a delayed ab-

[3] Supplied through the courtesy of The Texas Company, Houston, Texas.

sorption of water from the G-I tract of the adrenalectomized animals. Over six times as much water remained unabsorbed. However, this is not the major factor in respect to total water retention for, while approximately two-thirds of the ingested water was absorbed from the gut, very little was excreted. In contrast, the controls, which absorbed most of the ingested water, were able to excrete over one-half of it.

The distribution of the total water retained is shown in Table 2. The

TABLE 1. THE RETENTION OF WATER IN HYDRATED NORMAL AND ADRENALECTOMIZED RATS

	No.	Urine excreted cc./100 gm. ±S.E.	Total water retained in the animal cc./100 gm. ±S.E.	Total water retained in G-I tract cc./100 gm. ±S.E.	Total water absorbed but not excreted cc./100 gm. ±S.E.
Normal rats	18	6.2±0.4	4.6±0.3	0.6±0.2	4.0±0.2
Adrenalectomized rats	17	1.2±0.1	10.1±0.3	3.8±0.3	6.4±0.3

An average of 10.1 cc./100 gm. of water was administered. Total water retained was the sum of the increase in body weight plus the average weight loss during the two-hour period of the nonhydrated animals.

TABLE 2. THE DISTRIBUTION OF WATER IN THE INTACT RAT

		No.	Total body water cc./100 gm. ±S.E.	Expected total body water cc./100 gm.	Sodium space cc./100 gm. ±S.E.	Water retained in sodium space cc./100 gm.	Extra-sodium space cc./100 gm. ±S.E.	Water retained in extra sodium space cc./100 gm.
Normal rats	No water	24	71.7±0.3	—	34.6±0.3	—	37.1±0.2	—
	Water	18	76.0±0.5	76.3	35.6±0.4	1.0	40.4±0.4	3.3
Adrenalectomized rats	No water	28	72.0±0.2	—	32.6±0.3	—	39.5±0.2	—
	Water	17	81.8±0.4	82.1	35.7±0.4	3.1	46.2±0.6	6.7

total body water of nonhydrated adrenalectomized rats was not significantly different from their controls. Also, the total body water of both the hydrated control and adrenalectomized rats was very close to the expected value calculated on the basis of retained water.

The sodium space, which is an index of the extracellular fluid volume, decreased in the nonhydrated adrenalectomized rats, and since at the same time the total body water was unchanged, this implies a shift of water to the cells as was indicated by the increased extra-sodium space.

In the hydrated animals, most of the water retained was in the intracellular space. About three-fourths and two-thirds, respectively, of the retained water was located in the extra-sodium space of the control and adrenalectomized rats. The reason for this difference was due to the greater amount of water still in the gut of the adrenalectomized animal, which is part of the sodium space.

To determine the sites of absorbed but unexcreted water, it was necessary to measure the fluid compartment values of the gutless rat (Table 3). Total body water values of the gutless rats were slightly lower than those for the intact animals because of the high percentage of water in the gut of the latter. Again the total body water values for the hydrated animals are very close to the expected values calculated on the basis of the water absorbed but not excreted. As in the intact adrenalectomized animal, there was found an expected decrease in the sodium space, an increase in extra-sodium space, and no significant change in total body water thus indicating a shift of water to the cells. However, in the gutless animal nearly all

TABLE 3. THE DISTRIBUTION OF ABSORBED WATER IN THE GUTLESS RAT

		No.	Total body water cc./100 gm. ±S.E.	Expected total body water cc./100 gm.	Sodium space cc./100 gm. ±S.E.	Water retained in sodium space cc./100 gm.	Extra-sodium space cc./100 gm. ±S.E.	Water retained in extra-sodium space cc./100 gm.
Normal rat	No water	24	70.8±0.3	—	33.4±0.3	—	37.4±0.2	—
	Water	18	74.8±0.4	74.8	34.0±0.4	0.6	40.8±0.3	3.4
Adrenalectomized rat	No water	28	71.2±0.2	—	31.1±0.3	—	40.0±0.2	—
	Water	17	77.7±0.3	77.6	31.7±0.4	—	46.0±0.5	6.0

TABLE 4. ELECTROLYTE CHANGES IN NONHYDRATED AND HYDRATED NORMAL AND ADRENALECTOMIZED RATS

		No.	Serum Na m.eq./liter ±S.E.	Serum Cl m.eq./liter ±S.E.	Serum K m.eq./liter ±S.E.	Total sodium in 2-hour sodium space in m.eq./liter±S.E.		Radiosodium activity of gut in counts/second/gm. divided by counts/second/cc. of serum±S.E.
						Intact	Gutless	
Normal rats	No water	24	142.3±0.7	114.0±0.5	4.09±0.17	4.93±0.05	4.75±0.04	48.5±0.8
	Water	18	136.8±1.1	109.9±0.9	3.75±0.20	4.87±0.05	4.64±0.04	49.6±0.6
Adrenalectomized rats	No water	28	135.5±0.8	107.7±0.9	5.58±0.16	4.41±0.04	4.22±0.04	50.9±0.7
	Water	17	122.5±1.4	97.6±1.2	6.80±0.29	4.37±0.07	3.88±0.07	57.6±1.7

of the retained water was found to be located in the extra-sodium space. This was particularly true in the case of the adrenalectomized animal in which over ninety per cent of the absorbed but unexcreted water had shifted into the cells.

To determine what might account for these fluid shifts, certain changes in electrolyte balance were determined. As indicated in Table 4, a decrease in serum sodium and chloride and an increase in serum potassium occurred in the adrenalectomized rats, as was to be expected. Likewise, there was also a significant decrease in the total sodium in the two-hour sodium space. In the nonhydrated adrenalectomized animals, there was shown a slight increase in the radiosodium activity of the gut which was significant at the five per cent level according to Fisher's "t" test. This suggests a possible impairment in the absorption of diffused radiosodium from the gut. An in-

crease of this magnitude, however, would not be significantly reflected in the sodium space.

In the hydrated adrenalectomized animals, the serum sodium and chloride are reduced still further by dilution, except for potassium, which for some reason increased. Nagareda and Gaunt (1951) have shown, however, that there may be an increased output of adrenal cortical hormones in excessively hydrated rats; and since these hormones have been considered to regulate the distribution of potassium between the tissues and the blood plasma, the increased serum potassium of the hydrated adrenalectomized rats may be due in part to the absence of this hormonal mechanism (Ingle *et al.*, 1937; Marenzi, 1938).

The total sodium content of the intact animals did not change significantly during hydration. The total sodium of the gutless animals, of course, decreased because the hydrated guts had a larger sodium space than those that were nonhydrated. The radiosodium activity of the gut from hydrated adrenalectomized rats was much higher than that of the hydrated controls, but this was due primarily to the increased sodium space of the gut. Thus, it was not possible, with the methods used, to detect specifically a decreased rate of sodium absorption from the gut of hydrated animals during adrenal insufficiency, although this would be expected from the work of other investigators (Clark, 1939; Stein and Wertheimer, 1942).

DISCUSSION

The normal diuresis which follows excessive hydration has been considered due to the decreased plasma osmotic pressure which inhibits the release of the antidiuretic hormone from the posterior pituitary (Chambers *et al.*, 1945; Verney, 1946, 1947). Recent investigation has also suggested that a large water load in the rat stimulates the release of adrenal cortical hormones which inhibit the renal tubular reabsorption of water (Nagareda and Gaunt, 1951; Boss *et al.*, 1950).

Therefore, the absence of the adrenal cortical hormones during adrenal insufficiency plus the increased sensitivity of the renal tubules to an accumulating amount of an antidiuretic substance in the serum, provides the primary reason for water retention in the hydrated adrenalectomized rat.

Gaunt (1944) has demonstrated that a deficiency in the intestinal reabsorption of water and sodium salts may play an extra-renal role in regard to water retention in hydrated adrenalectomized rats. He also reported a shift of salt from the extracellular fluid to the unabsorbed water in the gut of such animals. This decrease in the electrolyte concentration of the extracellular space would result in a shift of water to the cells and a consequent increase in the intracellular space.

Shipley (1945), however, was unable to show a marked delay in the intestinal reabsorption of water in hydrated adrenalectomized rats. He also concluded, on the basis of thiocyanate volume and total body water determinations, that the greatest portion of the absorbed but unexcreted water was retained almost entirely in the extracellular fluid compartment.

This study has indicated that a delayed reabsorption from the gut does play an extra-renal role in connection with water retention during adrenal insufficiency. Although it was not established that a retention of sodium salts in the gut play a role in this regard, the results do not deny such a possibility. It has been observed in this laboratory that two hours after the oral administration of radiosodium to hydrated rats, the serum activity is significantly higher in normal animals than in those that were adrenalectomized. While this suggests an impaired absorption of sodium in adrenalectomized rats, it could be partially due, however, to a sluggish gastric motility which would lessen the opportunity for reabsorption.

This investigation also shows that the absorbed but unexcreted water was retained almost entirely in the cells of the adrenalectomized animals. The changes in electrolyte balance suggest the reason for this fluid shift. There was over a ten per cent loss of total sodium from the two-hour sodium space in the two-day adrenalectomized rat. In an endeavor to maintain the extracellular body fluids in a state of isotonicity, there was a compensatory loss of fluid from the extracellular space. This was detected as a decrease in sodium space. Since the total body water did not change during the two-day period of adrenal insufficiency, the fluid shifted into the intracellular compartment. Because the serum sodium, however, did decrease during this two-day period, it was assumed that loss of salt and the subsequent fluid shifts were still in a dynamic state of change. Proportionally, more of absorbed but unexcreted water shifted to the cells in the hydrated adrenalectomized animals than it did in their controls, probably because the loss of extracellular salt produced a higher osmotic gradient.

Since water intoxication is generally considered to be due to cellular hydration, these observations help to explain the sensitivity of adrenalectomized animals to the excessive ingestion of water. It has been suggested (Hays and Mathieson, 1945; Birnie *et al.*, 1948) that the extrarenal protection which adrenal steroids afford to adrenalectomized rats in this respect is very likely due to their effect on the distribution of water between extracellular and intracellular compartments, to prevent excessive intracellular hydration.

The present study even more strongly emphasizes this possibility and consequently further experiments have been designed to test this hypothesis.

SUMMARY

Normal and two-day adrenalectomized rats received two doses of water (5 cc./100 gms.) an hour apart. Two hours after the first dose, the adrenalectomized rats had retained over twice as much water as their controls. This retention was due in part to a delayed intestinal absorption, but primarily, to a delayed water diuresis.

The absorbed but unexcreted water was found to be located almost entirely in the cells. The loss of salt from the extracellular fluid during the two-day period of adrenal insufficiency was suggested as the cause of the increased cellular hydration in these animals.

The tendency for fluid to shift to the cells during adrenal insufficiency may explain in part the sensitivity of the adrenalectomized animal to excessive hydration.

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FAILURE OF THE DINITROPHENOL-INDUCED FALL IN PLASMA PROTEIN-BOUND IODINE TO STIMULATE AUGMENTED TSH PRODUCTION[1]

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THE fall in circulating protein-bound iodine induced by thyroidectomy or by antithyroid agents results in hypertrophy and hyperplasia of anterior pituitary basophils, and this is presumed to reflect increased TSH production. That this is not an invariable result, however, is brought out in the present study with dinitrophenol (DNP). DNP administration depresses the concentration of protein-bound iodine in plasma of the rat, but affects neither the anterior pituitary basophils nor the size and histological structure of the thyroid gland.

EXPERIMENTAL

In experiments 1 and 2, male rats of the Long-Evans strain, weighing from 200 to 220 gm., were used. They were maintained on a stock diet (Taurog and Chaikoff, 1946a) which contained 0.1γ iodine per gram. Female Sprague-Dawley rats weighing 190 to 200 gm. were used in experiments 3 and 4; these had been fed Purina Laboratory Chow which was found to contain about 1γ of iodine per gram.

The experimental design is shown in Table 1. In the first two experiments, recrystallized 2,4-dinitrophenol was dissolved in distilled water and brought to a final concentration of 5 mg. per cc. at pH 7.4. 16 rats received 1 cc. intraperitoneally every 8 hours for 21 or 30 days.

In experiments 3 and 4, DNP was mixed with ground Purina Laboratory Chow to the extent of 0.15 per cent. Fifteen rats were maintained on this diet for 30 days. Ten rats received 0.25 per cent methylthiouracil (MTU) in their drinking water (experiment 4).

At the end of the experiments, the rats were anesthetized with sodium pentobarbital. Blood was withdrawn by heart puncture, and determinations of protein-bound iodine were carried out by a procedure described elsewhere (Taurog and Chaikoff, 1946b, 1946c). Representative portions of heart, liver, kidney, and thyroid glands were fixed in 10 per cent neutral formalin. Paraffin sections were stained with hematoxylin-eosin and with Masson's trichrome method. The pituitaries were fixed in sublimate-formol (9:1), sectioned at 2μ, and stained by a modification of the method of Martins (1933). Quantitative cytological analysis of the pituitary was carried out as described elsewhere (Goldberg and Chaikoff, 1950).

Received for publication July 10, 1951.

[1] Aided by a grant from the U. S. Public Health Service.

[2] Atomic Energy Commission Fellow (National Research Council).

TABLE 1. THE EFFECT OF DNP ON THYROID GLAND SIZE, PLASMA PROTEIN-BOUND IODINE AND THE ANTERIOR PITUITARY

Expt.	Number of rats	Treatment		Days	Thyroid gland		Plasma protein-bound iodine	Anterior pituitary*			
		Dinitrophenol	MTU#		Weight	Histological appearance		Basophil	Acidophil	Chromophobe	Appearance
			per cent		mg. per 100 gm.†		γ per cent#	per cent	per cent	per cent	
1	10	None	None	Control	9.4±0.5	Normal	3.8±0.2	5.7	37.6	56.7	Normal
	6	15 mg. IP daily	None	21	8.5±0.3	Normal	1.4±0.1	4.2	39.4	56.4	Normal
2	10	None	None	Control	9.7±0.4	Normal	3.6±0.2	6.3	38.4	55.3	Normal
	10	15 mg. IP daily	None	30	8.3±0.5	Normal	1.5±0.3	5.7	36.7	57.6	Normal
3	5	None	None	Control	6.9±0.4	Normal	3.1±0.2	6.1	31.2	57.7	Normal
	5	0.15 per cent diet	None	30	6.7±0.6	Normal	1.6±0.2	5.3	37.4	57.3	Normal
4	5	None	None	Control	11.1±0.7	Normal		6.1	40.0	53.9	Normal
	5	0.15 per cent diet	None	18	8.3±0.3	Normal		5.0	42.6	52.4	Normal
	5	None	0.25	18	19.6±1.2	Hypertrophy, hyperplasia		14.4	29.8	55.8	Enlarged, vacuolated basophils. Degranulation of acidophils.
	5	0.15 per cent diet	0.25	18	17.2±1.3	Hypertrophy, hyperplasia		12.7	34.3	53.0	Enlarged, vacuolated basophils. Degranulation of acidophils.

Methylthiouracil in drinking water.
* Counts are averages of at least 2000 cells per gland.
† Average value with standard deviation.

RESULTS

I. The Response of Thyroid and Anterior Pituitary to Dinitrophenol Administration

Thyroid gland.—No increase in thyroid weight (Table 1) and no histological changes in this gland were noted in any of the DNP-treated rats. No cytological indications of increased thyrotrophic stimulation, such as increased acinar cell height, vascularity, intracellular colloid droplets, mitotic activity, or vacuolization of colloid were observed, nor was a reduction in follicular colloid noted. As judged by these criteria, the thyroids of the DNP-treated rats appeared even less active than normal.

Anterior pituitary.—Cytological examination of the anterior pituitaries of the DNP-treated rats revealed neither hypertrophy nor hyperplasia of the basophils. Quantitative analysis of the various cell types in these rats showed no increase in the percentages of basophils.

Microscopic examination of the other organs gave no indication of pathological alterations.

Plasma protein-bound iodine.—The levels observed (Table 1) were reduced to the same extent as that reported earlier (Wolff, Rubin and Chaikoff, 1950).

II. Response of Thyroid and Pituitary of Dinitrophenol-treated Rats to Goitrogen Administration

The finding that the anterior pituitary failed to respond to the fall in protein-bound iodine induced by DNP led us to test the capacity of this gland to react to goitrogen administration. The results of such an experiment are recorded in Table 1. It is clear that the simultaneous administration of DNP and methylthiouracil results in an increased percentage of basophils and the typical goitrous response of the thyroid gland.

DISCUSSION

Hypertrophy and an increase in the number of pituitary basophils are sensitive measures of thyrotrophic hormone production. Thus, Griesbach, Kennedy, and Purves (1949) observed that the daily administration of 2.4 γ dl-thyroxine per 100 gm. body weight prevented changes in the basophils in the thyroidectomized or goitrogen-treated rat, whereas the administration of 2.2 γ dl-thyroxine resulted in a significant elevation in the numbers of these cells.

Our failure to find a change in the pituitary basophils in the DNP-treated rats, together with the absence of either thyroid enlargement or histological manifestations of heightened glandular activity, indicates that the basophils were not secreting increased amounts of TSH. Since it has

been suggested that DNP affects phosphorylations in cells (Loomis and Lipmann, 1948; Cross *et al.* 1949; Teply 1949), the failure of the basophils to respond to the lowering of plasma PBI induced by the administration of this compound might be construed to result from an inhibition of some enzymatic system in the basophils. That this is not the case, however, is shown by the finding that the basophils of the DNP-treated rat still respond to the administration of a goitrogen.

The findings presented here suggest that the level of circulating thyroid hormone is not the only factor controlling TSH production by basophils. In view of the influence of DNP on the level of cellular metabolism, it is conceivable that another factor controlling TSH production is this metabolic level, an effect perhaps mediated through the hypothalamus.

We are indebted to Drs. A. Taurog and J. Wolff for iodine determinations.

SUMMARY

1. The administration of 2,4-dinitrophenol results in a marked decline in the level of plasma protein-bound iodine.

2. Cytological study of the anterior pituitary and thyroid glands of DNP treated rats failed to reveal changes indicative of increased TSH production.

3. It is suggested that the level of circulating thyroxine is not the only factor regulating TSH production.

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A DIFFUSION METHOD FOR THE ESTIMATION OF FORMALDEHYDOGENIC STEROID*

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ONE method for the estimation of cortical hormone in urine is the determination of the formaldehyde produced by the oxidation of the ketol side chain with periodic acid (Lowenstein, Corcoran and Page, 1946). In order to minimize the contribution of non-specific organic material, Daughaday, Jaffee and Williams (1948) developed a method in which the formaldehyde is distilled before it is estimated by means of the color reaction with chromotropic acid. Corcoran and Page (1948) have developed similar procedures.

It occurred to us that isothermal distillation of the formaldehyde by means of Conway cells (Conway, 1947) would be useful in this determination. One such method has been reported by Bassil and Hain (1950) but is inconvenient since it requires three to four days to reach equilibrium and does not permit quantitative recovery. The solution of this problem is to allow the distillation to occur directly into the chromotropic acid reagent, which is dissolved in 15 M concentrated sulfuric acid, thus converting the Conway cell to a small desiccator. The complete distillation of the aqueous formaldehyde solution permits the dissociation of the hydrate.

EXPERIMENTAL

Reagents and apparatus:

Glacial Acetic Acid: Distilled over chromic acid.

Chloroform: Redistilled reagent grade chloroform.

Ethanol, aldehyde free: 95% alcohol distilled over 0.1% 2,4-dinitrophenylhydrazine.

Periodic acid: 0.003 M solution stored in ice box.

Stannous chloride reagent: This is prepared fresh daily by dissolving 2 gm. stannous chloride in 5 ml. 0.1 N hydrochloric acid. Solution is effected more readily if the stannous chloride has previously been ground in a mortar and pestle.

Received for publication July 16, 1951.

* This study was supported by grants from the Damon Runyon Memorial Fund for Cancer Research, the American Cancer Society, the National Cancer Institute of the National Institutes of Health, Public Health Service, and the Atomic Energy Commission Contract No. AT(30-1)-910.

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Chromotropic Acid Reagent: 0.2% in 15 M sulfuric acid. The chromotropic acid is purified by treating an aqueous solution of Eastman Kodak Practical Grade Chromotropic Acid with charcoal and then recrystallizing from aqueous acetone. The final reagent is stored in the icebox.

Desoxycorticosterone.

Conway diffusion cells, porcelain: Obtainable from A. H. Thomas Co., Philadelphia.

Coleman spectrophotometer, junior model.

Test tube cuvettes 9×100 mm. for the spectrophotometer are fitted with ground glass stoppers so that the colors can be directly developed by heating the cuvettes in the water bath.

Procedure for Urine

Urine is collected in clean bottles without the use of preservative and stored in the refrigerator. For analysis in duplicate, a 4–5% aliquot of an exact 24 hour specimen of normal urine is taken. If the urine is abnormal, a volume is extracted such that it will yield 15–90 gamma of formaldehydogenic steroid.

The urine is brought to pH 1 with the addition of concentrated sulfuric acid and allowed to stand at room temperature for 24 hours.

The urine is extracted twice with double its volume of chloroform. The combined chloroform extract is washed once with 10% of its volume of ice cold 0.1 N NaOH and twice with 10% of its volume of water. Each washing is back extracted with 50% of its volume of chloroform. The combined chloroform extract is evaporated in vacuo to a small volume in an all glass still at 45° C. The extract is then taken up in aldehyde free alcohol and transferred to a 15 ml. centrifuge tube. The alcohol is evaporated off under a stream of nitrogen or air while heating the tube to 40° C. Any solid material adhering to the side of the tube is washed down with a small quantity of alcohol and this solvent is again blown off. The tubes containing the samples are left overnight in an evacuated desiccator over concentrated sulfuric acid.

The sample is wet with 0.10 ml. glacial acetic acid and after standing ten minutes, 1.50 ml. 0.002 M periodic acid reagent is added with shaking. The oxidation is allowed to proceed for one hour at room temperature at the end of which time the reaction is stopped with 0.40 ml. of the stannous chloride solution.

0.40 ml. aliquots of the reaction mixture are then pipetted into the outer well of a Conway cell and 0.80 ml. chromotropic acid reagent placed in the inner well. The previously greased cells are sealed by means of stiff stopcock grease and distillation allowed to proceed in the dark, overnight or for about fifteen hours. The contents of the inner well are then transferred to a small glass stoppered test tube and heated in a boiling bath for thirty minutes in the absence of light. This transfer does not have to be quantitative. The tube is then cooled in ice, allowed to come to room temperature and read at 570 Å in the spectrophotometer. To prepare a blank, a solution containing 0.02 ml. glacial acetic acid, 0.38 ml. water, and 0.80 ml. chromotropic acid is heated in the water bath along with the samples. The spectrophotometer is adjusted to zero optical density with this blank. Duplicate samples should agree within 5%. It is also necessary to do a reagent blank in which distilled water is substituted for urine and the entire extraction and distillation procedure gone through as outlined above. This blank value is subtracted from the values obtained for the standards and urine extracts.

Calculation

A standard curve is prepared by oxidizing known amounts of desoxycorticosterone under the same conditions as the urinary extract is oxidized. This curve is reproduced

here as Figure 1. Results on urine are expressed as milligrams of desoxycorticosterone excreted in twenty-four hours.

RESULTS

Diffusion of Formaldehyde from Pure Solution:

A solution of formaldehyde was so diluted that 0.1 ml. when heated with a mixture of 0.3 ml. of water and 0.8 ml. chromotropic acid would give an optical density of about 0.40 when read against a chromotropic

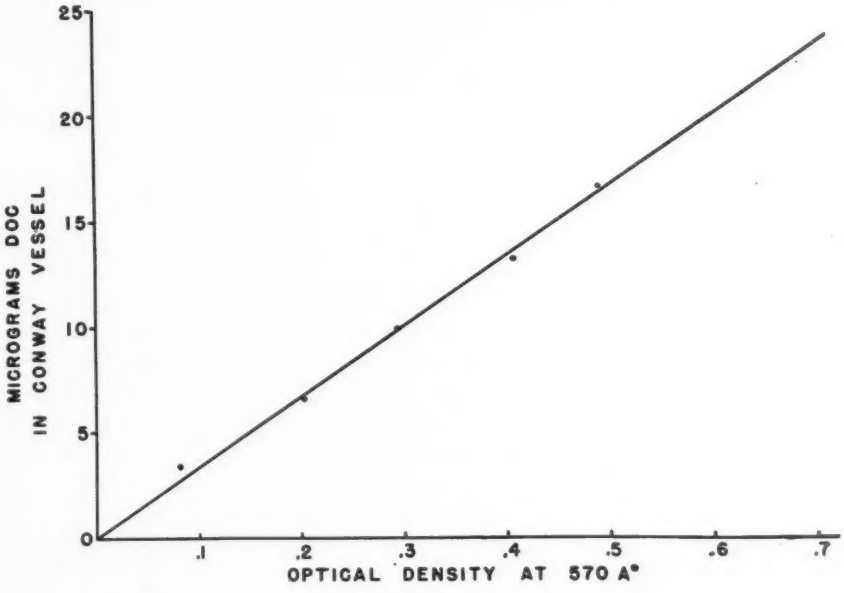

FIG. 1

acid blank. 0.1 ml. aliquots of this formaldehyde solution were mixed with 0.2 ml. periodic acid reagent and 0.1 ml. stannous chloride solution in the outer well of the Conway cells while 0.8 ml. chromotropic acid solution was placed in the inner cup. It required about 8 hours for all the aqueous solution to distill into the sulfuric acid at room temperature. This was necessary to insure complete dissociation of the hydrate of formaldehyde as well as to determine the final volume of the chromotropic acid solution. At various intervals the contents of the inner cup were transferred to glass stoppered test tubes which were heated in a boiling water bath for 30 minutes, then cooled and read at 570 Å against a chromotropic acid-water blank. These values were compared to those obtained by allowing mixtures of comparable amounts of formaldehyde and chromotropic acid to

stand at room temperature for a length of time equal to that used in the diffusion sample after which they were heated to develop the color, Table 1.

It is apparent that distillation of formaldehyde is almost complete when all the water has distilled, which at 25° C. requires about 8 hours. However, in our experience we have found it more reliable to wait a few hours longer than that. Since setting up the cells in the late afternoon is quite convenient, it is our practice to develop the colors the following morning.

TABLE 1. RECOVERY OF FORMALDEHYDE BY DIFFUSION

Time of diffusion Hours	Optical density Distilled Formaldehyde	Optical density of standard prepared by direct mixing of formaldehyde and chromotropic acid
8	.39	.45
11	.40	.44
17	.39	.40
24	.39	.39
36	.36	.39

Comparison with Distillation Method:

Fifty gamma samples of desoxycorticosterone were oxidized with HIO_4 essentially as in the method of Corcoran and Page (1948). Suitable aliquots of the distillate were taken and analyzed for formaldehyde by heating with chromotropic acid. The diffusion method gave a result which agreed within 5% of that obtained by distillation.

Studies on Normal Urine:

Exact 24 hour specimens were analyzed from laboratory personnel. Table 2 summarizes the results.

Recovery of Added Steroid to Urine:

Compounds E or F were added to fifteen different samples of urine. Table 3 shows the wide variations in recovery observed under these conditions.

We learned from Dr. Harold Mason, of the Mayo Clinic (1951) that formaldehydogenic steroid could not quantitatively be recovered after addition to urinary extracts. We then studied the recovery of formaldehyde added to urinary extracts and found it to be quantitative. Urinary extracts were then partially purified by Girard separation into ketonic and non-ketonic fractions, as well as by distribution between benzene and water, resulting in a benzene soluble and water soluble fraction. Table 4 shows that formaldehydogenic steroid added to three crude urinary extracts was recovered to the extent of 56, 58, and 82%. Recovery from the

water soluble material obtained from benzene-water partition varied from 75–100%. Recovery from the ketonic fraction of urinary extracts varied from 86–100%. The poor recoveries from the non-ketonic fractions and the benzene soluble fractions are demonstrated.

DISCUSSION

The present 24 hour room temperature hydrolysis at pH 1 may have to be modified in the light of the demonstration by Paterson, Cox, and Marrian (1950) that there is an acid labile corticosteroid released quickly from acidified urine and a more acid stable steroid released rather slowly.

TABLE 2. FORMALDEHYDOGENIC STEROID IN NORMAL URINE

Age	Sex	Mg./24 hrs.
24	M	.46
		.42
28	M	1.00
		1.12
33	M	.82
		.83
60	M	.75
		.71
30	M	.64
		.71
24	F	.54
		.59
27	F	1.00
		.93
39	F	.50
		.54
21	F	1.03
		.98
60	F	.38
		.35
70	F	.21
		.41
30	F	.34
		.39
60	F	.23
		.25
50	F	.98
		1.05

The extraction procedure uses two volumes of chloroform to one of urine because in this manner troublesome emulsions are avoided. We have been unsuccessful in attempting continuous chloroform extraction because of thick emulsion formation. In our experience the distillation of chloroform removes some non-specific chromogen.

The thermal distillation step by present methods makes it quite difficult to do large numbers of analyses in a working day. The diffusion procedure greatly simplifies this. We routinely analyze twenty-five urinary extracts in duplicate in five laboratory hours.

The diffusion procedure also allows the final color to be developed in a

much smaller volume than by present methods. From Figure 1 it can be seen that 3 micrograms of formaldehydogenic steroid can be measured. We are at present working out an ultra-micro modification of this precedure which should improve the sensitivity by a factor of ten.

Urinary extracts when subjected to thermal distillation yield material

TABLE 3. RECOVERY OF COMPOUND E AND F FROM URINE

Steroid added	Steroid recovered	Recovery	Conditions of recovery
gamma	gamma	%	
100 F	73	73	F added to urine
100 F	40	40	F added to urine
100 E	90	90	E added to urine
100 E	15	15	E added to urine
50 E	17	34	E added to urine
50 E	25	50	E added to urine
50 E	30	60	E added to urine
50 E	46	92	E added to urine
50 E	30	60	E added to urine
77.5 E	51	66	E added to urine
77.5 E	46	59	E added to urine
77.5 E	48	62	E added to urine
77.5 E	23	29	E added to urine
77.5 E	57	74	E added to Hydrolysate

TABLE 4. RECOVERY OF ADDED COMPOUND E FROM CRUDE AND PURIFIED URINARY EXTRACTS

E added	Crude	Benzene-water partition		Girard-separation	
		Water-soluble fraction	Benzene-soluble fraction	Ketonic fraction	Non-ketonic fraction
gamma	%	%	%	%	%
72		88	75	93	
72		92	61	97	61
72		100		98	
72	58	75	73	100	60
72	56				
72	82				

which is chromogenic on reaction with chromotropic acid. Corcoran and Page (1948) corrected for this by doing a blank estimation on a urinary extract treated with Stannous chloride prior to periodic acid. By allowing the dried extracts to equilibrate in a vacuum desiccator over concentrated sulfuric acid, any volatile material which will distill into sulfuric acid is removed. This step theoretically makes unnecessary such a blank. Under our particular conditions, when we did attempt to estimate this blank, we found the values were not reproducible and were roughly proportional to the amount of formaldehydogenic steroid present. For these reasons we abandoned the "unoxidized blank."

Isothermal distillation eliminates the occasional poor color obtained with the thermal distillate of an oxidation mixture from a particularly bulky urinary extract.

We have not been able to completely eliminate the reagent blank. Although the concentration of periodic acid is lower than that used in present methods, it still produces some chromogen with pure acetic acid. Under our conditions this gives a reagent blank of approximately 0.02 optical density when read against the acetic acid, water, and chromotropic acid mixture in the spectrophotometer. Most of this color comes from the acetic acid.

Our normal values for urine are approximately those reported by other workers (Daughaday, *et al.*, 1948 and Corcoran and Page, 1948).

In spite of the evidence that added steroid is not quantitatively recovered when added to urine, and that recovery is better from the water soluble extract after benzene water partition, and excellent from the ketonic fractions, we have not added these procedures to our routine method of measuring formaldehydogenic steriod. The mechanism of loss of formaldehydogenic steroid in crude fractions seems related to some inhibitor of periodic acid oxidation which on purification goes into the non-ketonic fraction, or the benzene fraction after benzene-water partition. We have no clue as to the chemical nature of this inhibitor.

SUMMARY

A method for the rapid estimation of large numbers of samples for formaldehydogenic steroids has been developed utilizing the diffusion of the formaldehyde into chromotropic acid.

Acknowledgement

The authors wish to express their appreciation to Dr. Konrad Dobriner for his helpful suggestions.

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HYPERPLASIA AND I^{131} UPTAKE OF THE GUINEA PIG THYROID DURING AND AFTER CHRONIC PROPYLTHIOURACIL TREATMENT*

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THE use of radioactive iodine and of chemical antithyroid agents in experimental studies on the thyroid gland has added considerably to present day understanding of the histophysiology of this organ. The early studies of Hertz and his colleagues (1939, 1941) and of Hamilton and Soley (1940), in which radioiodine was employed for the first time in thyroid investigation, ushered in a technic which enabled more precise interpretation of the relationship between iodine metabolism, the thyroid and the hypophysis. The observation by these investigators that hyperplastic thyroids collected more radioiodine than normal was followed by the converse demonstration of LeBlond and collaborators (1940, 1941); namely, that the radioiodine uptake of the thyroid gland in the hypophysectomized animal is considerably reduced. Using tracer doses of radioactive iodine, Morton, Perlman and Chaikoff (1941) found that thyroids activated with thyrotophin rapidly converted iodide to organically-bound iodine compounds in the gland; the appearance of these labeled compounds in the circulation was also determined. These basic observations have since been confirmed and extended (Morton *et al.*, 1942; Cortell and Rawson, 1944; Randall and Albert, 1951, and others). The demonstration by the Mackenzies (1943) and Astwood, Sullivan *et al.* (1943) that drugs of the aminobenzene and thiocarbonamide series inhibited thyroid hormone synthesis, resulting in functional hypothyroidism with thyroid hyperplasia, offered the means whereby sequential reactions of iodine to thyroid

Received for publication July 16, 1951.

* This investigation was supported, in part, by research grant RG-2407, National Institutes of Health, Public Health Service.

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hormone in the gland could be dissociated and studied. The failure of thyroid hyperplasia to occur with such substances in the absence of the hypophysis implicated the thyrotrophic mechanism of the anterior pituitary in the thyroid enlargement, as had previous studies on goitrogenesis in which rape seed diets had been used (Griesbach, Kennedy and Purves, 1941). Subsequent work on numerous species has fully confirmed these findings (review; Charipper and Gordon, 1947).

Although investigations utilizing antithyroid agents or radioiodine proceed intensively, there are relatively few experiments in which both have been used, and these have been restricted primarily to the rat and chick. Goitrogens markedly reduce radioiodine collection by the thyroid in these species (Franklin, Lerner and Chaikoff, 1944; Rawson *et al.*, 1944; Larson *et al.*, 1945a; Vanderlaan and Vanderlaan, 1947; Vanderlaan and Greer, 1950), and results of clinical studies are consistent with these findings (Stanley and Astwood, 1947; Keating *et al.*, 1950). Radioiodine studies have been carried out in the guinea pig, but the relative refractoriness of this species to antithyroid agents (Mackenzie and Mackenzie, 1943; Williams, Weinglass and Kay, 1944) has apparently discouraged its use in studies of goitrogenesis. The oral administration of thiourea, however, eventually induces morphologic changes in the guinea pig thyroid (McQuillan and Trikojus, 1946) and propylthiouracil in the ration causes thyroid hyperplasia which can be correlated with increased titers of blood TSH (D'Angelo, 1950). No experiment has been reported, however, in which the radioiodine uptake of the guinea pig thyroid has been measured and correlated with anatomic changes in the gland during and after goitrogen treatment; moreover, the few experiments of this nature in the rat and chick were not carried beyond 2 or 3 weeks. The present report describes the radioiodine collection and hyperplastic changes of the thyroid gland during and after chronic propylthiouracil treatment (2–160 days).

MATERIALS AND METHODS

Immature female guinea pigs (250–300 gm. body weight) procured locally were fed the normal laboratory ration consisting of purina (omolene) chow, supplemented with ample amounts of greens. The iodine content of the diet (100 μg./100 gm.) together with that in the tap water insured adequate iodine intake. Animals were used for the experimental study only after consistent body weight increased had been established. The propylthiouracil was administered in the basic purina ration (0.1%). The actual daily intake of drug per animal was not measured but was estimated to be 75–150 mg. daily, as calculated from the amount of ration consumed by groups of animals over a weekly period. Goitrogen was given for periods ranging from 2 to 160 days. Control animals received identical treatment except for the absence of the drug in the basic ration. At selected intervals throughout the experimental period, groups of animals were removed from the drug regime and returned to the normal diet for 5 days before sacrificing them.

The radioactive iodine[5] (I^{131}) was given intraperitoneally. Each animal received a single tracer dose (without carrier) of 8 μc. in 1 ml. solution. Twenty-four hours after radioiodine administration the guinea pigs were lightly anesthetized and 8–10 ml. of blood were withdrawn by cardiac puncture from each animal for bioassay experiments. The animals were then sacrificed by a sharp blow at the base of the head and the thyroid lobes were quickly removed, trimmed and weighed on a microtorsion balance. The right thyroid lobe was digested in 3 ml. of 2% NaOH for 72 hours prior to determination of its radioactivity and the left lobe was fixed in Bouin's picro-formol solution for subsequent

TABLE 1. HYPERPLASIA AND I^{131} COLLECTION OF THE THYROID GLAND IN FEMALE GUINEA PIGS DURING AND AFTER CHRONIC PROPYLTHIOURACIL TREATMENT

Days on (off) drug	Mean body wgt. (gm.) at sacrifice	No. pigs	Thyroid wgt. / body wgt.	Radioactivity of thyroid: Per mg. tissue (c/s)	(% change of normal)	Entire gland (%)
0	333 ± 21[1]	6	15.8 ± 0.9[2]	7.14 ± .57[3]		2.9
2	308 ± 23	4	16.8 ± 0.6	1.08 ± .17	(−84.8)	.4
2; (5)	325 ± 13	4	15.2 ± 1.1	8.68 ± 2.20	(+21.5)	3.0
0	377 ± 43	9	15.1 ± 1.2	4.85 ± .41		2.6
5–8	405 ± 43	5	15.4 ± 1.2	.59 ± .12	(−87.8)	.4
8; (5)	341 ± 22	5	15.5 ± 1.0	8.33 ± 1.50	(+71.7)	4.0
0	379 ± 26	5	17.8 ± 1.1	4.74 ± .45		2.8
15–18	324 ± 42	5	14.8 ± 2.9	1.76 ± .30	(−62.8)	.7
13; (5)	323 ± 17	3	15.7 ± 1.3	8.07 ± 1.30	(+70.3)	3.7
0	338 ± 22	6	15.6 ± 1.0	7.14 ± .58		2.9
23–29	348 ± 24	6	24.5 ± 2.8	2.03 ± .30	(−71.6)	1.2
24; (5)	351 ± 34	3	17.9 ± 2.3	9.45 ± 2.10	(+32.3)	3.5
0	497 ± 137	3	14.7 ± 4.0	4.78 ± 1.00		3.1
47*	523 ± 52	3	45.5 ± 12.4	.93 ± 0.44	(−80.5)	1.4
0	556 ± 98	8	12.9 ± 1.0	2.69 ± .45		2.7
60–84	548 ± 61	8	62.5 ± 11.3	.89 ± .10	(−66.9)	4.0
55–79; (5)	599 ± 54	5	46.1 ± 6.8	5.97 ± 1.40	(+121.9)	23.6
0	703 ± 76	6	14.1 ± 1.0	3.53 ± .58		2.4
100–160	541 ± 64	4	151.5 ± 28.6	1.13 ± .22	(−67.9)	7.2
95–156; (5)	617 ± 67	5	112.7 ± 3.5	9.45 ± 1.79	(+167.7)	47.5

[1] Mean Body Weight ± average deviation.
[2] Mean Thyroid Weight (mgm. ± standard error of mean) per 100 gram Body Weight.
[3] Mean # of counts per second ± standard error of mean.
* Withdrawal of goitrogen not tested at this time interval.

histologic examination. After homogenization in NaOH, 0.2 ml. of the thyroid suspension was pipetted onto a copper planchette and dried. The radioactivity of the sample was then measured in the Geiger counter as counts per second and was compared with that in an aliquot of the original sample injected into the animals. From these values, the amount of radioactivity per mg. of wet thyroid tissue and the percentage of the administered I^{131} which accumulated in the gland were calculated.

[5] The radioactive iodine was supplied by the Atomic Energy Commission, Oak Ridge, Tenn.

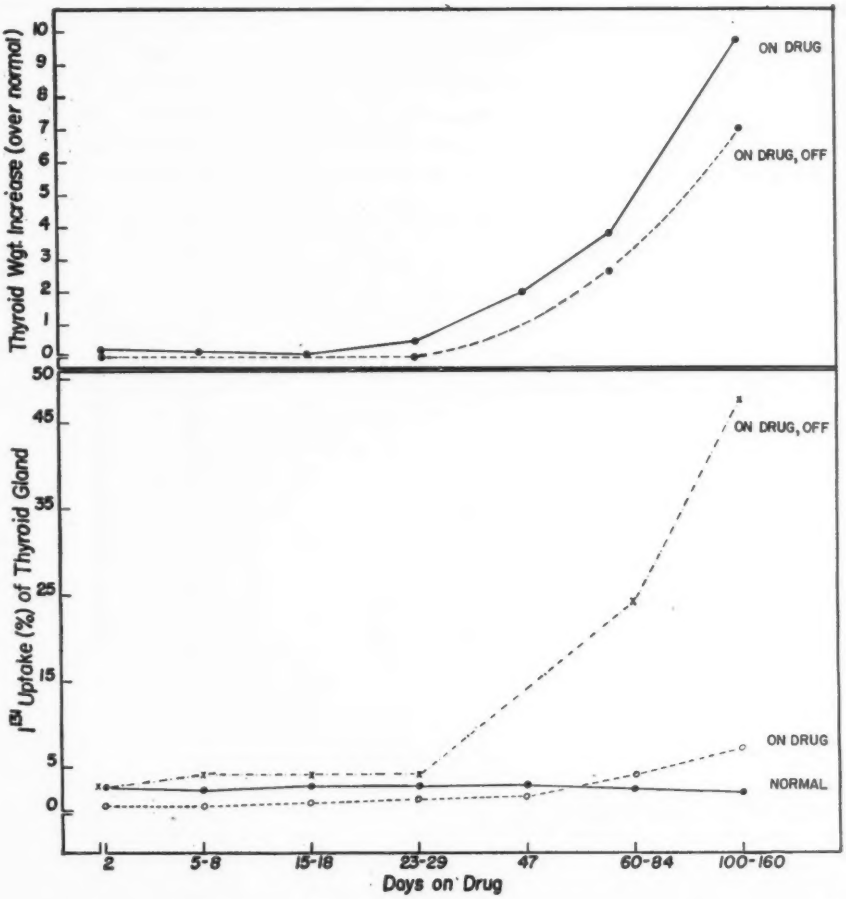

FIG. 1. The upper graph illustrates the course of thyroid hyperplasia (expressed as unit fold increase in weight) with uninterrupted propylthiouracil treatment (solid line), and after drug withdrawal (broken line). Curves in lower graph depict % uptake of I^{131} by the whole thyroid gland in animals on drug throughout (broken line), in those after goitrogen withdrawal (dot-dash), and in controls (solid line).

RESULTS

Administration of propylthiouracil in the ration eventually induced marked hyperplasia of the thyroid gland in the female guinea pig (Table 1, Figure 1). Although the thyroid did not become goitrous until after 3 to 4 weeks of treatment, enlargement of the gland appeared to be progressive thereafter. Thyroid weight increases approximating 1000 per cent of

normal were found at the 100–160 day interval, with no indication at this time that hyperplasia had reached a maximum. There was slight histologic change in the thyroids of some animals after 8 days of goitrogen administration but the morphologic changes were uniformly evident only after 15 to 18 days of treatment. These early histologic alterations, involving increased vascularity, increase in the height of the acinar epithelium, and

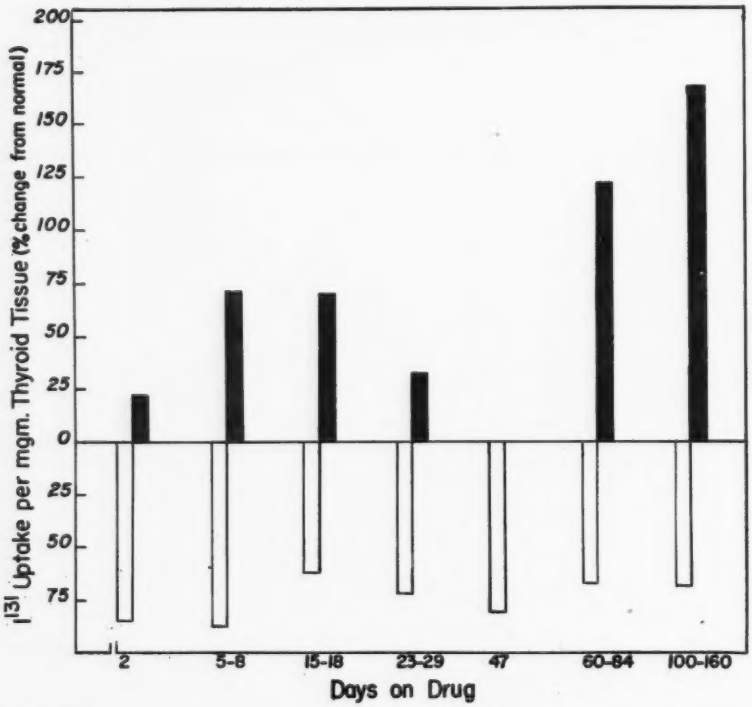

FIG. 2. Open and solid bars represent, at each time interval, depressed and augmented radioiodine uptakes per mg. of thyroid tissue, respectively, in animals continuously drugged, and in those after drug withdrawal. Values are expressed as % change from control uptake values.

colloid resorption occurred long before significant weight increases in the gland. They became accentuated as treatment continued.

Treatment with the antithyroid agent apparently altered thyroid gland activity before any appreciable anatomic changes were visible in the gland. Marked depression of radioiodine collection was found as early as 2 days after propylthiouracil administration and there were significantly lowered

I^{131} concentrations (per mg. of wet thyroid tissue) in the glands of all animals maintained without interruption on the drug regime (Table 1, Figure 2). The depression in iodine uptake was just as marked with short as with prolonged periods of treatment. Despite the fact that iodine collection was uniformly decreased throughout the period of drug administration, the total radioactivity in the gland increased progressively from sub-normal values to exceed eventually the total radioiodine collection in normal thyroids. These augmented radioiodine accumulations were largely a reflection of the increased thyroid mass inasmuch as the efficiency of thyroid tissue for capturing the I^{131} (as evidenced by the concentration per mg.) was impaired almost equally throughout. It was not until thyroid enlargement was substantial (5 to 10 fold), however, that the total iodine accumulation in the glands of treated animals exceeded that in normal controls.

The I^{131} collection of the thyroid gland increased appreciably when animals which had received the drug for varying periods of time were returned to the normal diet. Not only did the depressed uptake values return to normal levels but values were attained which were significantly above normal. This "rebound" phenomenon seemed to depend in part upon the duration of previous treatment with the goitrogen, the degree of rebound paralleling generally the course of thyroid hyperplasia (Figure 1). The increased iodine uptake resulted not only from increased tissue mass, however, but also from increased avidity of thyroid tissue to fix iodine (Figure 2). Some evidence of this increased capacity for radioiodine accumulation was found after only 2 days of drug treatment. It was well defined in thyroids of animals given the goitrogen for 8 days before return to the normal diet; in these, radioiodine concentrations were approximately 72% above that in normally fed animals. The efficiency of thyroid tissue in collecting I^{131} became greater the longer animals were maintained on propylthiouracil before withdrawal of the drug. The radioiodine uptake (per mg. thyroid) was 121.9% of normal after 8 to 10 weeks on the goitrogenic diet, and was still increasing at the 13 to 22 week interval (167.7%). This increased avidity, together with the greatly increased mass of tissue, resulted in total radioiodine accumulations in the gland equivalent to approximately one half the total amount injected into the animal. Since the total I^{131} uptake under such circumstances represented augmented function as well as increased tissue mass, it was to be expected that the increase in radioiodine accumulation would be greater than the degree of hyperplasia attained by the thyroid.

The microscopic changes in the thyroids of animals maintained without interruption on the goitrogen for prolonged periods were of extreme degree. The hyperplastic glands were characterized by a highly vascularized,

solid epithelial mass in which the original follicular arrangement was discernible only at the periphery. Colloid was practically absent except for weakly-staining shreds. In central portions of the gland, lumina were so obliterated that the follicular epithelium assumed cord-like formations. The acinar epithelium was generally tall columnar and contained either swollen, or condensed, irregularly-shaped nuclei. The return of goitrous animals to the normal diet for 5 days effected profound morphologic changes in these hyperplastic thyroids. The glands decreased in size, although still remaining markedly enlarged (Table 1). Considerable amounts of deeply-staining colloid were deposited in the gland and the follicular arrangement was restored. The microscopic appearance of these glands was far from normal however. The irregularly-shaped lumina were usually traversed by islands of large epithelial cells. These papillary invasions, together with heavy connective tissue septa, imparted a honey-combed, lobulated appearance to the gland. In contrast, the histologic changes in thyroids of animals maintained for short periods (2 to 8 days) on drug before withdrawal were not striking.

DISCUSSION

Except for the delayed onset, the morphologic changes induced in the guinea pig thyroid with propylthiouracil appear identical with those obtained in other species treated either with antithyroid agents or with exogenous thyrotrophin. There is little reason to doubt that the thyroid hyperplasia produced by goitrogens results from unrestrained secretion of thyrotrophic hormone following inhibition of thyroid hormone formation (Mackenzies, 1943; Astwood *et al.*, 1943). Increased blood titers of TSH and basophilia of the anterior pituitary have been demonstrated in guinea pigs rendered goitrous by prolonged propylthiouracil treatment (D'Angelo, 1950). The relative ease with which thioureylenes induce thyroid hyperplasia in the chick and rat, as contrasted with the guinea pig, may well indicate differences in lability of the thyrotrophic mechanism of the pituitary in these species, inasmuch as there appears to be no correlation between the "resting" level of the thyroid and its response to goitrogenic agents. The chick and guinea pig thyroid are relatively inactive histologically, whereas the rat thyroid appears hyperactive. On the other hand, blood titers of TSH are high in the rat and the bird (pigeon) but low in the guinea pig (D'Angelo and Gordon, 1950).

The studies of Keating and colleagues (1945, 1945a, 1945b) have shown that thiouracil inhibits radioiodine accumulation in normal chick thyroids as well as in those made hyperplastic with TSH, and these investigators have correlated the radioactive iodine uptake with anatomic changes in

the gland after such treatment. Radioiodine uptake by the rat thyroid is also diminished by thiouracil (Franklin *et al.*, 1944; Rawson *et al.*, 1944) or propylthiouracil (Vanderlaan and Vanderlaan, 1947; Vanderlaan and Greer, 1950), with the latter substance more effective than the former in decreasing thyroidal I^{131} uptake in both species (McGinty *et al.*, 1948). The results of the present study reveal that the radioiodine collection of the guinea pig thyroid is also markedly depressed by goitrogens. The prolonged period of treatment made it possible to demonstrate that propylthiouracil can effectively inhibit I^{131} uptake throughout 5 to 6 months of uninterrupted treatment. The marked depression in uptake found at the 2 day interval of treatment suggests that the inhibitory effect on the thyroid, as in other species, occurs rapidly and before appreciable anatomic changes become visible.

Despite uniformly decreased radioiodine uptake with chronic propylthiouracil treatment, the total radioactivity in the thyroid eventually exceeds normal when sufficient hyperplasia has occurred to offset the limited uptake. The augmented radioiodine collections which result when the drug is withdrawn, however, are much more considerable, and are greater than would be expected from the hyperplasia alone. Short term experiments on the chick and rat have indicated that the diminished radioiodine uptake of enlarged thyroids quickly rises to exceed normal after goitrogen withdrawal. Larson *et al.* (1945a) considered this rebound phenomenon to reflect increased thyroid mass rather than hyperfunction. It is clear from the present study that this effect cannot be entirely accounted for by the hyperplastic changes in the thyroid and must result in part from increased avidity of thyroid tissue for the radioactive iodine. This augmented avidity for iodine, upon withdrawal of the drug, is demonstrable after periods of treatment too short to have caused hyperplasia, although it increases progressively with longer periods of treatment (and, consequently, with thyroid hyperplasia) before the goitrogen is withdrawn.

The precise mechanism responsible for the rebound phenomenon of I^{131} uptake in the guinea pig thyroid is not known. Certain previous observations may possibly be related to this effect. LeBlond and Sue (1941) noted that in the guinea pig thyroid, stimulated for several days with exogenous TSH, augmented uptake of I^{128} was due chiefly to increased efficiency of cells rather than to cellularity. The substantially increased thyroidal I^{131} uptakes found after withdrawal of the goitrogen may possibly indicate a response to endogenous thyrotrophin. The manner in which endogenous TSH could induce the increased avidity for iodine may possibly involve the iodide concentrating mechanism of the thyroid. It is now evident that the ability of the thyroid gland to concentrate iodide does not depend on

its capacity to form thyroid hormone (Astwood, 1944–45). There is suggestive evidence that the normal thyroid has an iodide concentrating mechanism independent of subsequent conversion to di-iodotyrosine or thyroxine (Taurog, Chaikoff, and Feller, 1947). In acute propylthiouracil experiments (Vanderlaan and Vanderlaan, 1947), the anatomically unaltered thyroid of the rat concentrates iodide normally although incorporation into organic compounds is reduced; continued treatment augments this iodide concentrating activity to a greater degree than it does thyroid enlargement. The rapid formation of bound iodine in such glands after goitrogen withdrawal fails to occur if the hypophysis is removed even though the thyroid remains hyperplastic for days; accordingly, Vanderlaan and Greer (1950) postulate the existence of an enzyme system, sensitive to the absence of TSH, which is responsible for the iodide-trapping function of the thyroid. The present study yields no direct information regarding the activity of an iodide concentrating mechanism in the guinea pig thyroid, nevertheless, the rebound effect may well reflect some such type of mechanism. It is conceivable that prolonged administration of propylthiouracil may have enhanced iodide concentrating capacity while thyroid hormone synthesis remained blocked by the drug. Upon withdrawal of the goitrogen, the ability of the thyroid to bind iodine organically would be quickly regained and probably accentuated since the now hyperactive iodide concentrating mechanism would facilitate greater delivery of iodine to the synthetic process. As a result, the increased uptake of iodine would be greater than could be accounted for by the increased thyroid mass. That augmented organic binding of iodine may have occurred after withdrawal of the goitrogen was strongly suggested by the fact that the thyroid hormone activity of the blood was increased above normal when tested in the stasis tadpole (D'Angelo, unpublished). It must be emphasized that there are other possible explanations for the rebound phenomenon of radioiodine uptake and that the one proposed here is hypothetical at present.

SUMMARY AND CONCLUSIONS

The chronic administration of propylthiouracil to the guinea pig induced marked hyperplastic changes in the thyroid gland, with nine to tenfold weight increases occurring after 100–160 days of uninterrupted treatment. The I^{131} uptake by the thyroid (per mg. of wet tissue) was decreased appreciably in animals receiving the goitrogen. The degree of depression in I^{131} accumulation was as marked with short (2 days) as with long periods (160 days) of treatment and occurred before any anatomic changes were visible in the gland. Total radioactivity in the thyroid eventually exceeded

normal when sufficient tissue bulk had been attained to offset decreased uptake.

Radioiodine uptake was increased above normal upon withdrawal of the goitrogen. The "rebound" in uptake was greater the longer the period of previous treatment with drug, and paralleled the course of the hyperplasia. Increased avidity of thyroid tissue for radioiodine was demonstrable, however, before anatomic change in the gland. Augmented efficiency, together with increased tissue mass, resulted in radioiodine thyroidal collections 10 to 20 times greater than normal. Withdrawal of the goitrogen arrested the thyroid hyperplasia and resulted in considerable deposition of colloid.

The observations suggest that the increased avidity of thyroid tissue for I^{131} after withdrawal of the goitrogen cannot be accounted for entirely by anatomic changes, and, rather, may reflect change in a cellular mechanism, presumably enzymatic, in response to prolonged stimulation with endogenous thyrotrophic hormone.

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PRODUCTION OF DIABETES AND INCREASED ERYTHROCYTE SEDIMENTATION RATE BY PURIFIED GROWTH HORMONE

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IT HAS been demonstrated that highly purified growth hormone preparations may produce temporary (idiohypophyseal) diabetes in cats (Cotes, Reed and Young, 1949) and in dogs (Campbell, Davidson, Snair and Lei, 1950). It has also been possible to produce permanent (metahypophyseal) diabetes by highly purified growth hormone in dogs previously used as test animals in assays of diabetogenic materials (Campbell, Davidson and Lei, 1950).

In continuing these studies we have found that highly purified growth hormone greatly increased in dogs the erythrocyte sedimentation rate (ESR), decreased the volume per cent of red blood cells and appeared to increase the concentration of fibrin in the blood plasma. It is of considerable interest that these effects were most manifest when the diabetes induced by the growth hormone was also maximum.

METHODS

The rate of sedimentation of erythrocytes was determined in Wintrobe tubes which were suspended in a water-bath at 25° C. Potassium oxalate was used as anticoagulant. Readings were taken at 10 min. intervals for at least 1 hour, and as the sedimentation at 30 min. appeared to give the most representative values of the rates, the values are expressed as mm. sedimented in 30 min., the total height of the fluid column being 100 mm. The effect of the volume per cent of red cells on the ESR was determined in a number of blood samples from dogs, with widely different rates, by dilution of the blood with plasma, according to Rourke and Ernstene (1930). The total protein and fibrin of the plasma were determined by the methods of King (1946) and the sugar of blood and urine according to Somogyi (1945) and Benedict (1911) respectively.

The growth preparations were obtained in crystalline form from fresh bovine anterior pituitary glands by the method of Wilhelmi, Fishman and Russell (1948), via Fraction A. When assayed by the tibia method of Greenspan, Li, Simpson and Evans (1949), this material, W8, (Table 1) had activity equivalent to W4P, which was used in this test as a reference standard. Fraction W4P gave a growth response by the body-weight method (Campbell *et al.*, 1950) similar to that obtained for pure growth hormone by Li, Evans and Simpson (1949). It should be noted that in the above tibia tests on W8 and W4P, young hypophysectomized rats of the Wistar strain were kept at ordinary room temperatures. Subsequently hypophysectomized Sprague-Dawley rats were kept at a higher

Received for publication July 18, 1951.

and constant temperature (29-30° C). Under these conditions the responses to WP4 were uniformly higher than before over the dosage range of 12 to 216 μgm. in 4 days (Table 1). In these tibia tests a moist diet of whole milk, brown bread and ground lean meat in the proportions, by weight, of 2.5:1:2 was given.

The ACTH content of W8, assayed by the "difference" method of Sayers, Sayers and Woodbury (1948), was about 2%. Previous results (Campbell *et al.*, 1950) indicate that this amount of ACTH would not be expected to have an appreciable effect on the diabetes produced by the growth hormone. When a similar preparation (W5) was administered (5 mg. during 4 days) to young hypophysectomized rats there was evidence (stimulation of the thyroid epithelium) that a small undetermined amount of thyroid-stimulating hormone was present.

EXPERIMENTAL RESULTS

Prior to the experiments described, pituitary diabetes had been produced in each of the 3 dogs by the administration of a partially purified globulin fraction of the anterior pituitary gland for 6 days, in order to test for sensitivity to the diabetogenic effects of the extract. Following these injections, the signs of diabetes disappeared in 1 to 2 days, and a month elapsed before beginning the injections of the growth hormone fraction.

The 3 male dogs were given subcutaneously 1.75 mg. crystalline growth hormone preparation per kg. twice daily. The injections were given for 15 consecutive days, then, after an interval of 5 days without injection, Dogs BT and T were injected for 9 days, while Dog J was injected for 21 days. Thereafter Dog J remained permanently diabetic. Food was given twice daily, the diet per day being: ground lean meat 300, "Purina" fox chow 100 and sucrose 40 gm. The initial body weights were 12.9, 12.4 and 8.6 kg., respectively, and by the 30th day had increased by 3.3, 0.8 and 1.7 kg.

Stage of idiohypophyseal diabetes

The administration of growth hormone produced severe diabetes in the 3 dogs, as is shown in Figure 1. After a short lag phase, pronounced hyperglycaemia and glycosuria occurred, followed by a gradual diminution which indicated the development of resistance to the diabetogenic effect of growth hormone. In the interval of 5 days without injection these signs of diabetes disappeared. On resuming the injections for 9 days, the diabetogenic responses in Dogs BT and T were definitely less than had been elicited before, although the dosage was the same. Re-administration of the growth hormone to Dog J caused a lesser rise in blood sugar during the first 8 days. However, by continuing the injections great hyperglycaemia and glycosuria were elicited. After growth hormone had been given for a total of 36 days this dog became permanently diabetic.

The administration of growth hormone to the 3 dogs greatly increased the sedimentation rate of the erythrocytes, as is shown in Figure 1. The

increase, in general, corresponded with the severity of the diabetes. In the 5-day interval without injection the ESR declined to approximately normal values. On resuming the injections for 9 days the ESR again rose in the 3 dogs, but not to the same extent as before. The more prolonged administration of growth hormone to Dog J greatly increased the ESR throughout the remainder of the injection period. Under these conditions

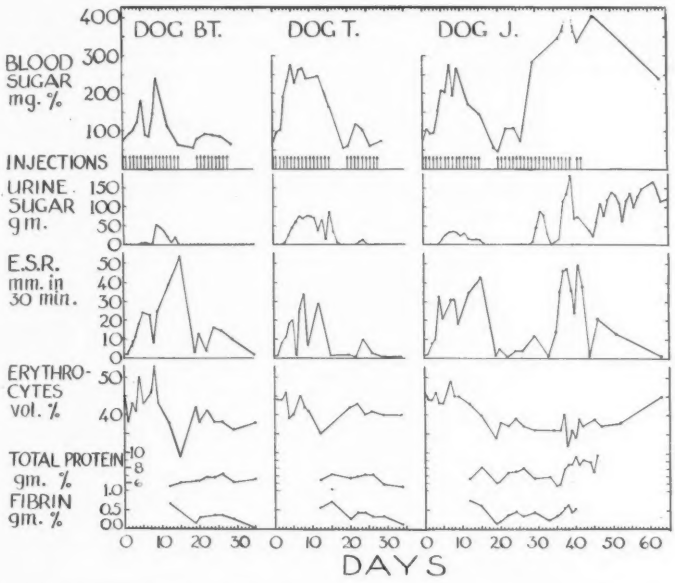

FIG. 1. The changes in sugar excretion, in the sugar, erythrocyte sedimentation rate (ESR) and haematocrit readings of blood, and in the total protein and fibrin of plasma in male dogs given growth hormone preparation W8. Each arrow represents the injection of 3.5 mg. W8 per kg.

the increase in ESR corresponded approximately with the hyperglycaemia and glycosuria.

There were occasions, within the first 9 days of injection, when the haematocrit readings were above the normal level, but for the most part the readings were definitely below normal (Figure 1). Even during the 5 days without injection the readings remained low. The data of the experiments on dilution of blood by plasma showed that these low volumes per cent of red cells could account for only a small part of the total rise in ESR.

There are indications (Figure 1) that the levels of total protein and fibrin were higher when injections of growth hormone were given than when

they were withheld, but it would be unwise to draw final conclusions from these limited data.

On the last 6 days of injection, visible lipaemia occurred in the plasma of Dog J. This has been observed in other dogs during the height of the diabetes produced by the injection of growth hormone.

Stage of metahypophyseal diabetes

The injection of growth hormone produced permanent diabetes in Dog J. For 2 months following the last injection, averages of over 2 liters of urine and well over 100 grams of sugar were excreted daily, with morning

TABLE 1. ASSAY OF GROWTH-PROMOTING ACTIVITY

Growth activity was assayed by the tibia test (Greenspan *et al.*, 1949). Female rats (75 to 80 gm.) were hypophysectomized and, after 14 days, the test preparations, in 0.5 ml. saline, were injected daily, intraperitoneally, for 4 days. The width of the proximal uncalcified cartilaginous disc of the tibia was measured 24 hours after the last injection.

Date	Product	No. of rats	Total dose μg. per rat in 4 days	Response width of tibial cartilage	
				μ	Increase, μ
4/ 7/50*	W4P	5	216	249	126
		4	72	224	101
		6	12	166	43
	W8	3	100	240	117
		4	40	196	73
	Controls	5	0	123	0
23/10/50**	W4P	6	216	322	153
		6	72	281	112
		6	12	222	53
	Controls	6	0	169	0

* Wistar rats, ordinary room temperatures.
** Sprague-Dawley rats, constant temperature, 29–30°C.

blood sugar values of 240 to 406 mg. per cent. The visible lipaemia, however, disappeared on ceasing the injections. Insulin administration was begun 2 months after the last injection and produced marked improvement in activity and apparent health. In the ensuing 9 months no change in the severity of the diabetes has been observed. Severe bilateral cataract developed about 4 months after the last injection of the growth hormone preparation.

In this permanently diabetic state the rate of erythrocyte sedimentation decreased from the previously high levels. On the 9th day after the last injection the ESR was still above normal (30 mm. in 30 min.), but by the 21st day it was normal and remained so thereafter (0 to 7).

In 5 female dogs which had been depancreatized and maintained on insulin for several months, the average ESR was above the normal, but of a lesser order of magnitude than that occurring in idiohypophyseal diabetes (Table 2). In these depancreatized dogs no correlation between blood sugar and ESR was evident. Possibly these sedimentation rates were largely influenced by the rather low haematocrit values.

In an untreated alloxan-diabetic dog of several months' standing the ESR was found to be normal (Table 2). Thus the great rise in ESR during idiohypophyseal diabetes was not seen in dogs with alloxan, pancreatic or metahypophyseal diabetes.

TABLE 2. ERYTHROCYTE SEDIMENTATION RATES OF DIABETIC DOG BLOOD

The depancreatinized dogs had been maintained on insulin for several months but the alloxan-diabetic dog was not given insulin

Dogs	No.	Sex	Blood		
			Sugar mg. per cent	Erythrocytes vol. per cent	Erythrocyte sedimentation mm. in 30 min.
Normal*	7 3	M F	61 to 80 56 to 73	45 to 56 46 to 68	0 to 1 1 to 3
Depancreatized	5	F	110 to 333	28 to 42	1 to 15 (Av. 5.8)
Alloxan-diabetic	1	F	278	42	1

* The total plasma protein averaged 5.99 gm. per cent, range 5.25 to 6.76; and the fibrin averaged 0.42, range 0.26 to 0.49.

DISCUSSION

The administration of adequate doses of a crystalline growth hormone prepared in this laboratory to fed dogs causes a progressive and marked increase in the rate of sedimentation of erythrocytes. The volume per cent of erythrocytes is usually decreased, but this cannot wholly account for the effect on the sedimentation rate. There is also a suggestion that the total protein and fibrin of the blood plasma increase. These changes coincide with, and follow fairly closely, the hyperglycaemia and glycosuria produced during the administration of the growth hormone preparation.

Such great changes in ESR, however, were not found during the subsequent metahypophyseal (permanent pituitary) diabetes produced in one of these dogs by growth hormone. In an alloxan-diabetic dog the ESR was normal, and in several depancreatized dogs was elevated, but not nearly to the same extent as by the administration of growth hormone. Thus it appears that hyperglycaemia from various causes is not always accompanied by a rise in ESR. Although these two effects were elicited by growth

hormone it cannot be concluded, from these experiments, that they are necessarily related.

While changes in plasma proteins and ESR have apparently not been recognized as characteristic signs of diabetes mellitus, the observations suggest that changes similar to those which occurred in the present experiments might be searched for in the various types of diabetes.

SUMMARY

Following recovery from a preliminary test in which temporary diabetes had been produced by the injection of a partially purified anterior pituitary extract, the administration of growth hormone to fed dogs produced definite sustained hyperglycaemia and glycosuria characteristic of idiohypophyseal diabetes. Prolonged administration of growth hormone produced permanent (metahypophyseal) diabetes in one of these dogs.

The administration of growth hormone increased the erythrocyte sedimentation rate (ESR) to very high levels. This effect corresponded approximately with the intensity of the idiohypophyseal diabetes. These values of ESR exceeded those found in metahypophyseal, pancreatic or alloxan diabetes.

The volume per cent of erythrocytes was, in general, reduced by the injections of growth hormone. This can account for only part of the total effect on the ESR. There are indications that the total protein and fibrin of the blood plasma were increased by the hormone administration.

Acknowledgments

We wish to acknowledge the encouraging interest and support of Professor C. H. Best in this work. Dr. D. Fraser generously made the depancreatized dogs available for study. The research was supported by a grant from the National Research Council of Canada.

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EFFECT OF GROWTH HORMONE ON MUSCLE AND SKIN COLLAGEN IN NEONATAL THYROIDECTOMIZED RATS

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GROWTH hormone administration produced marked body weight gain, muscle hypertrophy, increased skeletal dimensions, and skin thickening in hypophysectomized rats (Simpson *et al.*, 1949). All of these effects, with the exception of the muscle response, were observed in rats thyroidectomized at birth which were injected daily with growth hormone for 100 days (Scow, 1951a). The musculature of the treated animals was thin, pale, and flabby. The muscle weight gain was not great enough to overcome the characteristic muscle deficiency observed in thyroid deficient rats (Scow, 1951b). The marked weight response of skin and bone, both collagen containing tissues, suggested that the small weight gain in muscle might be a connective tissue response. The present paper reports studies on the collagen, as well as water and protein, content of muscle and skin in neonatal thyroidectomized rats treated with growth hormone.

EXPERIMENTAL

Thyroparathyroidectomy was performed on one day old Holtzman rats, both sexes, under refrigeration anesthesia (Scow and Simpson, 1945). All animals were maintained on lactating diet (#4164)[2] and greens (twice weekly). Daily intraperitoneal administration of growth hormone, 0.5 mg. Armour's preparation #22KRI[3] in normal saline solu-

Received for publication July 18, 1951.

[1] The author is indebted to Dr. E. E. Hay of Armour and Company for the supply of growth hormone, preparation #22KRI. Technical assistance of William C. Murphy is also gratefully acknowledged.

[2] Diet #4164 (Modification of McCollum's lactating diet (Scow and Simpson, 1945)). 67.5% ground whole wheat; 15% casein; 10% whole milk powder; 5.25% Crisco; 1.5% $CaCO_3$; .75% NaCl; Vitamin A 12,000 U.S.P. units and Vitamin D 2,150 U.S.P. units per 1 kilogram diet. (Protein 24.3%; carbohydrate 54.8%; and fat 11%.)

Diet #4185 (Scow, 1951b). 18% casein (vitamin free); 8% Wesson oil; 68.8% Dextrin; 4% Osborn and Mendel salt mix (J. Biol. Chem. **32:** 374. 1917); 0.18% $FeSO_4 \cdot 7H_2O$; 0.02% $CuSO_4 \cdot 5H_2O$; 0.03% concentrated cod liver oil; and 1% vitamin mixture (B complex and K).

[3] Armour's growth hormone preparation #22KRI contains minute amounts of thyrotrophic, adrenocorticotrophic and gonadotrophic hormones. Detailed findings of the

tion, was begun on the 35th day and continued for 100 days. Experimental procedure in more detail and other findings will be presented in another paper (Scow, 1951a). Another group of Holtzman rats, all females, was thyroparathyroidectomized at 25 days of age and sacrificed 60 days later. These rats were maintained on an 18% protein purified diet (#4185)[2] containing all the essential minerals and vitamins. One and one-half milligrams of Vitamin E was fed biweekly with a pipette (Scow, 1951b). These rats were not treated with growth hormone. The operated animals included in this paper were completely thyroidectomized as ascertained with the I^{131} radioautographic survey of Reinhardt (1942; Scow and Simpson, 1945).

Complete autopsies were performed at the conclusion of the experiments. The carcasses were then stored in moisture proof containers at subfreezing temperatures for several months before the chemical analyses were made. Water content was determined by drying the tissues to constant weight in a vacuum oven at 60° C. Nitrogen content was ascertained with macro-Kjeldahl digestion and micro-distillation of NH_3 on either the dried muscle sample, or an aliquot of an alkaline-alcohol hydrolysate of the skin (Kochakian *et al.* 1948). Collagen content was measured with the procedure of Lowry *et al.* (1941).

Great care was taken to obtain comparable muscle samples[4] from the individual animals for each test. The thigh muscle mass was used for the collagen and the leg muscle mass for the water and protein determinations. Collagen content of skin was determined on a 2–4 gm. unshaven sample from the middle of the back, and the other determinations were made on the remaining skin, also unshaven.

RESULTS

Thyroidectomy in young rats markedly impaired skeletal muscle growth (Table 1). The thigh muscle-body weight and leg muscle-body weight ratios were significantly less in thyroidectomized rats than in *ad libitum* fed or pair-fed normals. There was no difference in the collagen, protein and water content of muscle (% wet weight) between untreated thyroidectomized and *ad libitum* fed normal rats.

Growth hormone administration produced a marked body weight increase in neonatal thyroidectomized rats (Table 1). Although the absolute

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effect of the contaminants will be presented in another paper (Scow, 1951a). Their presence in the present experiments is considered unimportant for the following reasons: there was a complete absence of thyroid tissue; there was no acceleration of the retarded skeletal development observed in neonatal thyroidectomized rats or inhibition of body weight gain (Marx *et al.*, 1943) even though adrenal weight was appreciably increased; and the observed muscle deficiency and collagen response were of equal magnitude in both sexes, although gonadal secretion had been stimulated, as evidenced by the weight gain of accessory sexual tissues.

[4] The thigh muscle mass included the gluteal, hamstring and adductor muscles as well as the muscles having a femoral origin. The leg muscle mass included the calf muscles and those with tibial or fibular origin. The separation of the muscles from the skeleton was made by dividing the tendinous attachments as closely to the bone as possible. The dissected muscle mass included the related tendons, fascia, nerves and blood vessels, as well as muscular tissue proper.

weight of the muscle was increased, the subnormal muscle-body weight ratio was unchanged. The collagen content of muscle, either as per cent wet weight or per cent protein, was nearly doubled by the growth hormone, whereas the amount of water and protein (% wet weight) were unchanged.

Rats deprived of the thyroid gland at birth suffer marked retardation of skin and hair development. The infantile hair coat with soft, short, thin hair shafts, and the thin skin persist for a long period in the rat thyroidectomized at birth. (Greater details of this finding have been published earlier (Scow and Simpson, 1945)). The protein content of the skin

TABLE 1. EFFECT OF GROWTH HORMONE ON COLLAGEN CONTENT OF MUSCLE IN THYROIDECTOMIZED RATS

Treatment	No. and sex[a]	Body weight		Muscle (Unilateral)[b]					
				Thigh			Leg		
		Final gm.	Gain gm.	Weight % B. wt.	Collagen % wet wt.	Collagen % protein[c]	Weight % B. wt.	H_2O % wet wt.	Protein % wet wt.
135-day old Thyroidectomized at birth									
Growth hormone Injected	3 M	165	132± 17	1.96± .03	2.96± .14	13.9± 1.2	.86± .04	69.8± .7	21.5± .4
	3 F	145	115± 8						
Uninjected	1 M	64	27± 3	2.02± .12	1.18± .36	5.4± 1.5	.79± .05	69.9± 1.1	21.9± .3
	2 F								
Normal controls	3 M	426	—	4.00± .14	1.64± .14	7.2± .6	1.21± .02	72.3± .5	22.8± .4
	3 F	254							
85-day old Thyroidectomized at 25 days									
Uninjected	4 F	68[d]	—	2.36± .15	1.56± .05	6.6± .4	1.07± .05	74.4± .6	23.4± .3
Normal control Fed *ad lib.*	4 F	182[d]	—	4.40± .09	1.46± .08	6.4± .3	1.34± .04	74.1± .3	22.7± .4
Pair fed	6 F	69[d]	—	3.92± .10	—	—	—	—	—

[a] Sex differences were insignificant, so combined findings only are presented.
[b] Collagen as % protein: Collagen content of thigh is expressed as % of protein of leg. Protein calculated as 6.25×nitrogen.
[c] Mean± S.E. (S.E. = $\frac{\text{Range}}{\text{No. of rats}}$, Mantel, 1951).
[d] Fasted 17 hours.

(% wet weight) was much less than that of similar aged normals (Table 2). Collagen content of skin in neonatal thyroidectomized rats expressed as per cent wet weight was $\frac{1}{3}$ that of normal, whereas it was $\frac{2}{3}$ that of normal when expressed as per cent protein (the latter difference was not highly significant ($P < .05$) due to the small number of animals). In the 80-day old rat thyroidectomized at 25 days of age, the retardation of skin and hair development was less severe because of the more mature state of development at the time of thyroid extirpation. In these thyroidectomized rats there was more hair relative to the skin than in the neonatal thyroidectomized and normal control rats. This was reflected by the greater amount of protein (% wet weight) and smaller amount of collagen (% skin protein)

in the 80-day old thyroidectomized rats than in their normal controls (latter statistically significant at the level, $p = .02$).

Changes in the skin of growth hormone treated thyroidectomized rats were first noted around the sixth week when the skin became more difficult to penetrate with the hypodermic needle. Hair shafts were increased in cross section and length but did not appear to increase appreciably in number. The protein content (% wet weight) was significantly increased

TABLE 2. EFFECT OF GROWTH HORMONE ON COLLAGEN CONTENT OF SKIN IN THYROIDECTOMIZED RATS

Treatment	No. and sex[a]	H_2O %	Protein[b] %	Collagen	
				% Wet wt.	% Protein
135-day old Thyroidectomized at birth					
Growth Hormone Injected	3 M, 3 F	49.7 ± 2.9[c]	20.8 ± .8	14.9 ± .6	71 ± 4.3
Uninjected	1 M, 2 F	50.9 ± 4.8	14.2 ± 1.4	6.2 ± 1.2	42 ± 9
Normal control	3 M, 3 F	51.5 ± 1.1	28.3 ± 1.1	18.2 ± .4	64 ± 1.5
85-day old Thyroidectomized at 25 days					
Uninjected	4 F	42.6 ± 1.4	35.9 ± 1.8	18.6 ± 1.2	52.0 ± 3.8
Normal control	4 F	49.0 ± 1.8	31.4 ± 1.1	20.1 ± .6	66.5 ± 2.2

[a] Sex differences were insignificant, therefore combined findings only are presented.
[b] Protein = 6.25 × nitrogen.
[c] Mean ± S.E. (S.E. = $\frac{\text{Range}}{\text{No. of rats}}$, Mantel, 1951).

(Table 2). The collagen content per wet weight was doubled by growth hormone but was still subnormal. On the other hand, collagen content of skin expressed as per cent protein was greater in growth hormone treated thyroidectomized rats than in either untreated thyroidectomized or normal control rats, but the latter difference was of smaller magnitude and not statistically significant.

DISCUSSION

These experimental results clearly demonstrate stimulation of collagen formation in skin and muscle of thyroidectomized rats by the administration of growth hormone. Increased collagen content of skin was correlated with thickening of the corium layer. These findings are in agreement with those of Simpson *et al.* (1949) who reported great redundancy of skin,

"tougher" connective tissue, and "collagenous fibers . . . increased in size" (histological) in hypophysectomized rats injected with growth hormone for 437 days.

Collagen formation in muscle of growth hormone treated thyroidectomized rats occurred at the expense of other proteins, as indicated by its two-fold increase when expressed as per cent protein. The gain in muscle weight in response to growth hormone administration was proportional to that of the body weight, and thus did not correct the muscle deficiency so characteristic of thyroidectomized rats (Scow, 1951b). On the other hand, the muscle weight gain in growth hormone treated hypophysectomized rats was proportionally $1\frac{1}{2}$ times that of body weight (Simpson *et al.*, 1949). Since complete inactivity of the thyroid gland does not occur in the hypophysectomized rat, the difference in muscle weight response to growth hormone could then be attributed to thyroid hormone. Administration of thyroxin alone did not stimulate body growth in hypophysectomized rats (Smith, 1933; Evans *et al.*, 1939). These findings would then suggest that the muscular tissue (muscle cells) requires both thyroxin and growth hormone for its proper development.

Muscle mass makes up about 40% of the total weight of normal rats. A major part of the body weight gain in normal growing animals is then the result of wieght increase of muscle. The relative insensitivity of muscle to growth hormone in the absence of the thyroid hormone would then provide a plausible explanation for the small body weight gain observed in growth hormone treated thyroidectomized rats (Scow *et al.*, 1949; Ray *et al.*, 1950).

SUMMARY

Skeletal muscle growth was markedly impaired in rats thyroidectomized at birth or at weaning, resulting in a subnormal muscle-body weight ratio. However, the collagen, protein and water content (% wet weight) were normal. Growth hormone administration for 100 days to rats thyroidectomized at birth produced a two-fold increase in the muscle collagen (% wet weight or % protein), as well as a four-fold increase in body weight, but had no effect on the subnormal muscle-body weight ratio or protein and water content of muscle.

The skin of rats thyroidectomized at birth was very thin, the hair infantile and the collagen content, as well as protein, subnormal. Growth hormone administration to the rat thyroidectomized at birth produced a marked thickening of skin, an increase in the size of hair shafts and an increase in the collagen and protein content of skin (% wet weight). Although thyroidectomy in the weanling rat had less effect on skin and hair growth, the skin collagen as per cent protein was significantly subnormal.

Growth hormone appears to have little, if any, effect on the formation of muscular tissue in the absence of the thyroid gland.

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THE EFFECT OF TESTOSTERONE ON THE SEMINAL VESICLES OF CASTRATED, HIBERNATING HAMSTERS

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ALTHOUGH tissue growth during hibernation has been investigated to some extent (Sarnat *et al.*, 1942; Cassens, 1939), the response of a tissue in a hibernating animal to a controlled stimulus has not been studied. The following experiments were designed to compare the responsiveness of the seminal vesicles to injection of measured amounts of testosterone in castrated male hamsters in the hibernating state with similarly treated animals which did not hiberante.

MATERIALS AND METHODS

The testes were removed surgically from 16 adult male hamsters (Mesocricetus auratus). The hamsters were kept in the stock animal room for several weeks or months after castration to insure regression of the accessory sex organs to their basal condition. They were then transferred to individual cages in a cold room maintained at $5° \pm 2°$ C. (see Lyman, 1948, for details of maintenance). The hamsters hibernated at various times after being placed in the cold room. When deep in the hibernating state, they were injected subcutaneously with 0.2 cc of a suspension containing 5 mg of a long-acting testosterone propionate adsorbed on aluminum phosphate.[2] The injection procedure invariably aroused the hamsters for variable periods of time. They were observed daily for 10 days after the injection, and the number of days each animal was found in the hibernating state was recorded. As is shown in Figure 1, one animal hibernated for 9 days following the injection, one for 8 days, one for 7, one for 6/ four for 4, one for 3, and one for 2 days. Six animals did not resume hibernation during the 10-day observation period. Thus, of the sixteen animals, all were under the influence of testosterone for the full 10 days. Six of the hamsters were awake for the entire time after the injection, whereas in the remaining 10, the effect was exerted partly during the awake condition and partly during the hibernating state.

Ten additional castrated hamsters were maintained in the cold room but without injected testosterone. These animals constituted a control group from which the size and appearance of the seminal vesicles in their basal condition could be estimated.

At the end of the 10-day observation period, both control and injected groups of animals were killed. The seminal vesicles were removed and weighed on a Roller-Smith

Received for publication August 6, 1951.

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[2] This preparation was kindly supplied by Ciba Pharmaceutical Products Inc., Summit, New Jersey.

torsion balance. Representative samples of the glands were fixed in Bouin's fluid. After imbedding in paraffin, sections of 5μ thickness were cut and stained with hematoxylin and eosin.

RESULTS

The uninjected, castrated hamsters varied in body weight from 92 to 107 gms. when killed, with an average of 100.2 gms. Their seminal vesicles were small, varying from 46 to 100 mg., and averaging 64.8 mg. The six injected animals which did not return to the hibernating state showed a four-fold gain in the weight of their seminal vesicles. The body weights of

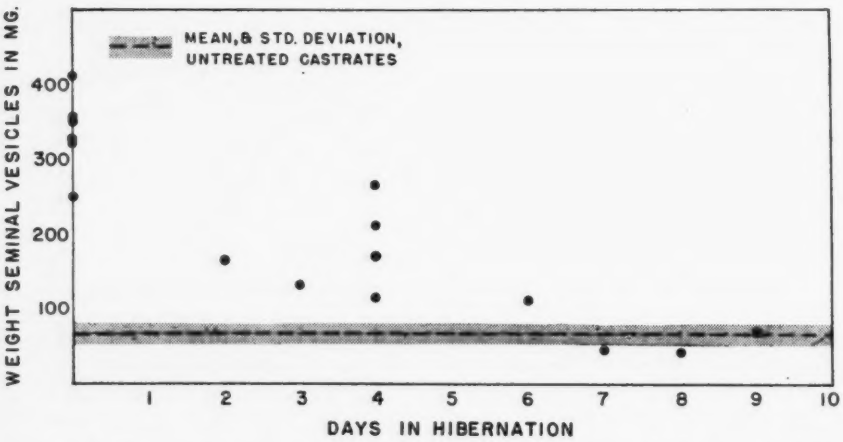

FIG. 1. The weight of seminal vesicles of castrated hamsters which hibernated for varying periods of time after injection of testosterone, compared with hamsters which did not hibernate. The mean and standard deviation of 10 untreated castrates provides a base line.

these hamsters varied from 82 to 95 gms., with an average of 86.5 gms., while the weights of their seminal vesicles varied from 249 to 410 mg. with an average of 344.3 mg.

The hamsters which returned to hibernation varied in body weight from 69 to 94 gms., with an average of 83.1 gms. The effect of testosterone on the seminal vesicles of these animals is shown in Figure 1. It is apparent that the three animals which hibernated for 7 days or more gave no evidence of a stimulating effect of testosterone, whereas the thirteen animals which either hibernated for less than seven days or did not return to hibernation at all showed increasing enlargement of their seminal vesicles.

Histologically, the seminal vesicles of the hamsters which hibernated for the longest periods after injection showed little evidence of stimulation, as illustrated in Figure 2. The lumens of the vesicles were small and filled with

a granular secretion containing desquamated cells and cellular detritus. The epithelial cells lining the glands were cuboidal or low columnar, with the nuclei situated near the center of the cells.

On the other hand, the seminal vesicles of the six hamsters which did not return to hibernation showed marked evidence of stimulation (Fig. 3). The lumens of the vesicles were greatly enlarged and filled with a faintly

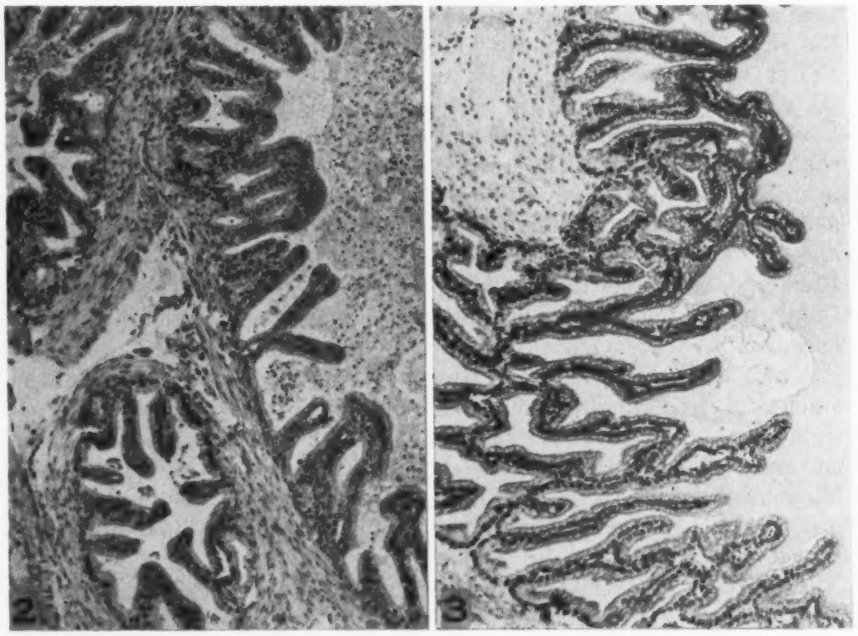

FIG. 2. Seminal vesicle of a hamster which resumed hibernation for 7 days following injection of testosterone. Wt. seminal vesicle—46.7 mg. ×100.

FIG. 3. Seminal vesicle of a hamster which did not resume hibernation following injection of testosterone. Wt. seminal vesicle—326 mg. ×100.

stained, homogeneous, acellular seminal fluid. The epithelial cells were columnar with basally situated nuclei. The supranuclear cytoplasm was clear (Golgi zone) with granular cytoplasm filling the distal ends of the cells.

It is of some interest that injected testosterone tends to curtail hibernation (Fig. 1). Previous experience has established that normal hamsters usually return to the hibernating condition within a day or two after being disturbed, and that an animal will hibernate even after such extensive manipulation as having wire electrodes attached into the skin of the back

(Lyman, in press). Furthermore, it was found in preliminary experiments that if hibernating hamsters were injected with more than 5 mgs. of testosterone, none of them returned to hibernation.

DISCUSSION

These experiments show that the hibernating state greatly reduces the response of the seminal vesicles to the stimulus of testosterone. Although the data presented here indicate that there is no response whatever when the animal is hibernating, this finding must be considered with caution. Foster (1934) has shown that female thirteen-lined ground squirrels (Citellus tridecemlineatus) breed less than five days after coming out of hibernation in the spring. Although Rasmussen (1917) found only slight changes in the testes of the woodchuck (Marmota monax) before waking in the spring, he did find (1918) that the ovary of the female underwent definite changes during hibernation. Though the growth rate of the reproductive organs might be so greatly decreased as to seem abolished in a 10-day period, longer times might reveal a slow, cumulative stimulation. In any event, the response of the target organs to stimulation by the sex hormones is apparently greatly decreased during hibernation.

A unique aspect of the hibernating state appears to be that it is not the amount of circulating hormone which determines the response of the affected organ, but rather the condition of the organ itself. Therefore, the endocrinological study of the sexual cycle of those species of rodents which hibernate must pose a problem not encountered in non-hibernating mammals, namely, that of the responsiveness of the organ in its different physiological states.

In previous papers (Lyman, 1948; Lyman and Hastings, in press; Lyman, in press) it has been emphasized that, in spite of a body temperature of 5° C., the hibernating rodent maintains a remarkably accurate control of certain vital processes. The present experiments emphasize that the concept of homeostasis in hibernation has definite limitations, and that there are other important aspects of the internal economy which remain almost completely in abeyance during the hibernating state.

SUMMARY

Castrated male hamsters were allowed to hibernate at an environmental temperature of 5° C. After the animals hibernated, they were injected with 5 mgs. of testosterone. Following this injection, some of the animals resumed hibernation, whereas others failed to do so. The seminal vesicles of these two groups were compared. The vesicles of the animals which returned to hibernation revealed little enlargement or histological changes, whereas those of the animals which remained awake showed marked in-

crease in weight and change of histological appearance. This reaction is considered unique because it is not the amount of circulating hormone which determines the condition of the affected organ, but rather the transient condition of the organ itself.

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THE SIGNIFICANCE OF THE GOMORI STAINING OF THE BASOPHILS OF THE RAT PITUITARY

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GOMORI (1950) has described an elastic tissue stain which also stains specifically certain of the "basophil" cells of the rat pituitary. Halmi, (1950) investigated the responses of the Gomori staining "basophils" to specific endocrine deficiencies. Purves and Griesbach (1951a) reported that the cells stained specifically by the Gomori stain in the rat pituitary corresponded with those "basophils" to which a thyrotrophic function has been ascribed (1951b). The evidence for the thyrotrophic function of this group of "basophils" was obtained in experiments correlating the glycoprotein content of the "basophil" cells as revealed by the staining method of McManus (1946) with the hormone content of the gland under conditions of specific endocrine stimulation or depression. In this publication evidence of the identity of the Gomori staining granules with the glycoprotein granules revealed by the McManus technique is presented. The functional responses of the Gomori staining granules to conditions affecting the thyroxine supply have been investigated. The results show that the Gomori staining granules in the rat pituitary are correlated with the contents of thyrotrophic hormone in a storage form.

METHODS

The pituitaries were fixed in formol sublimate solution (formalin 10 parts, saturated solution of mercuric chloride 90 parts). Sections were cut at a thickness of 3μ. Mercury compounds were removed from the slide by alcoholic iodine solution followed by thiosulphate in the usual way.

Various modifications of the Gomori stain were tried but none had any conspicuous advantage over the composition used by Gomori. It may be advantageous in some instances to increase the content of basic fuchsin. Doubling the concentration of hydrochloric acid gave much stronger staining reaction but the amount of non-specific staining was increased. The intensity of staining after formol sublimate fixation is greater than that obtained after Bouin fixation but with more non-specific staining. For most work the standard Gomori stain was used of the following composition:

0.5% solution of basic fuchsin in 70% alcohol—100 ml.,
conc. hydrochloric acid 1 ml.,
paraldehyde . — 1 ml.

Received for publication September 13, 1951.

The solution begins to stain specifically after three days ripening at room temperature. After staining half an hour in a jar the sections were differentiated with 1% hydrochloric acid in 70% alcohol until no further colour was removed.

For the glycoprotein staining, methods given in a previous paper (Purves and Griesbach, 1951b) were used.

For the evaluation of the numbers of Gomori positive cells in the pituitary, sections were cut in the horizontal plane. Sections were taken at a standard level in the pituitary, approximately 150μ from the inferior surface. At this level the pars intermedia and the beginning of the pars nervosa are visible at the anterior edge of the sections. The total number of Gomori positive cells in the whole section was counted.

RESULTS

Nature of the Gomori stained granules in the "basophils" of the rat pituitary.

The relation of the Gomori stained granules to the glycoprotein granules revealed by the McManus periodic acid Schiff method (PAS), was tested

FIG. 1. Section of rat pituitary showing cells stained by the Gomori stain. The cells are angular in outline and contain granules of varying size.

(Gomori stain; ×600).

by restaining by the PAS method pituitary sections which had been first stained by the Gomori stain. It was found that the Gomori positive cells were also PAS positive. The individual granules of the "basophils" stained with the Gomori stain were also stained by the PAS method, the mauve colour of the Gomori stain being overlain by the strong magenta colour of the PAS. There was no evidence of different granules in the basophil cells, some staining with Gomori and some with PAS. In order to obtain photographic evidence of the identity of the Gomori positive granules with the glycoprotein granules stained by PAS, the pituitary sections were first

stained weakly with a Gomori stain which had ripened for two days only. The selected fields were photographed using a green filter and process plates developed to a high contrast. The sections were then restained by the PAS method. The selected fields were then rephotographed. The intense magenta staining gave a high contrast image which gave a satisfactory negative with shortened development of the plate. There was thus obtained a photograph which showed the magenta colouration of the PAS stain, since the faint Gomori stain would, under these conditions, have been

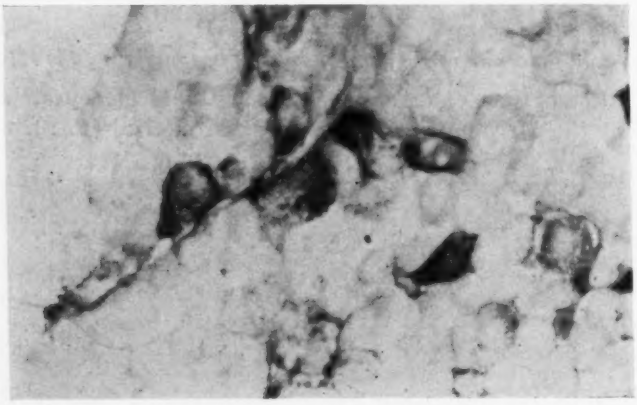

FIG. 2. Same section as Fig. 1. The same field rephotographed after staining with periodic acid-Schiff reagent. The Gomori stained granules are also stained by the PAS method showing them to be glycoprotein in nature. The only additional elements stained by the PAS are capillaries. For further details see text.

(PAS; ×600.)

at a very low level of contrast. This manoeuvre was necessary since it was not found possible to extract the Gomori stain from tissues once stained by it. Moreover, the Gomori stain is susceptible to mordanting action of variation of kinds and could not safely be applied to a pituitary which had been previously stained by PAS. The resulting photomicrographs are illustrated in Figures 1 and 2. It will be seen that the individual granules in the Gomori positive cells are reproduced faithfully in the photo of the PAS stained section. Certain additional elements are revealed by the PAS. In particular, the walls of the capillaries and the plasma inside the capillaries are prominent in the PAS stained section.

Relation of the Gomori staining to the two types of "basophils" in the rat pituitary.

The Gomori stain stains only those "basophils" which, on the basis of functional response, were considered to be thyrotrophic in function. The gonadotrophic "basophils" of the rat pituitary, which are located on the inferior surface and also in a zone adjacent to the intermedia, are not stained by the Gomori stain. In normal pituitaries stained by the Gomori

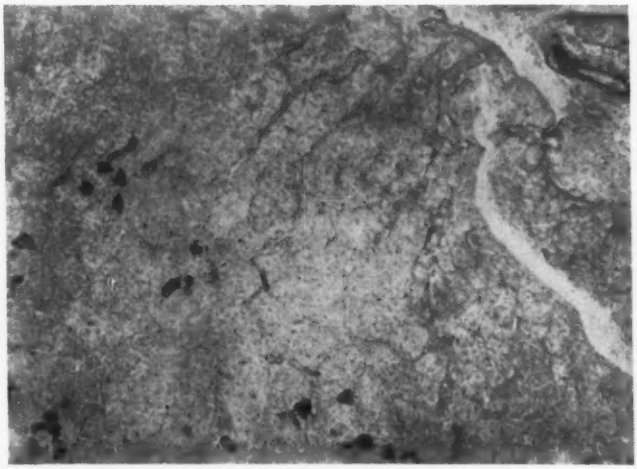

FIG. 3. Section of normal male rat pituitary showing region of anterior lobe adjacent to pars intermedia. At the top right-hand corner there is a portion of the pars intermedia containing a small artery, the elastica of which is stained black by the Gomori stain. Lower left there are some Gomori staining cells. The zone of the pars anterior adjacent to the pars intermedia is free of Gomori cells.

(Gomori stain; ×250.)

stain there was, therefore, a conspicuous zone in this region free of stained cells (Fig. 3). It is this zone which contains the majority of the gonadotrophic cells which stain strongly by the PAS method. These are best demonstrated by the PAS staining of the pituitary of the rat which has been treated with thyroxine. Under this treatment the thyrotrophic cells regress and lose their glycoprotein. The PAS stained gonadotrophs in such pituitaries occupy the zone which in the normal pituitary, is free of Gomori stained cells. (See Fig. 4.)

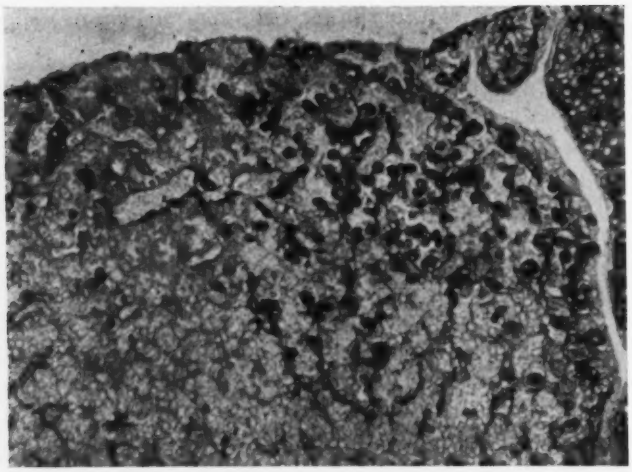

FIG. 4. Section of pituitary of male rat treated with L-thyroxine (10 μgs. per day) for one month. The same region is shown as in Fig. 3. Adjacent to the pars intermedia there is a dense collection of round or oval cells rich in glycoprotein which are gonadotrophic in function and occupy the zone which is free from Gomori cells in Fig. 2. The region in the lower left corner which would normally contain angular thyrotrophic cells is free of such cells since these have been inhibited by the thyroxine treatment.

(PAS; ×250.)

Response of Gomori cells to thyroxine deficiency.

The changes in the Gomori stained cells after thyroidectomy have been evaluated by counting the total number of cells per section as detailed under methods. Table 1 shows the number of such cells per section found at varying times after thyroidectomy. It will be seen that after 48 hours there

TABLE 1. EFFECT OF THYROIDECTOMY ON COUNTS OF GOMORI CELLS IN RAT PITUITARIES

Time after operation	No. of animals	Average Gomori cells per section	
		No.	% of normal
24 hrs.	4	1160	109.4
48 hrs.	4	280	26.4
72 hrs.	4	60	5.7
96 hrs.	2	67	6.3
120 hrs.	2	5	0.5
144 hrs.	2	3	0.3
168 hrs.	2	6	0.6
Unoperated	4	1060	100

is a very extensive loss of Gomori stained cells and that after five days the numbers of such cells are negligible.

TABLE 2. EFFECT OF L-THYROXINE INJECTIONS ON GOMORI CELLS OF THYROIDECTOMISED RATS. INJECTIONS WERE BEGUN ON THE DAY AFTER THYROIDECTOMY AND CONTINUED FOR 13 DAYS

Thyroxine dosage per 100 gms. per day	No. of rats	Average Gomori cells per section	
		No.	% of normal
Nil	10	0	0
1.5 μgs.	2	1045	99
3.0 μgs.	2	500	47
5.0 μgs.	2	340	32

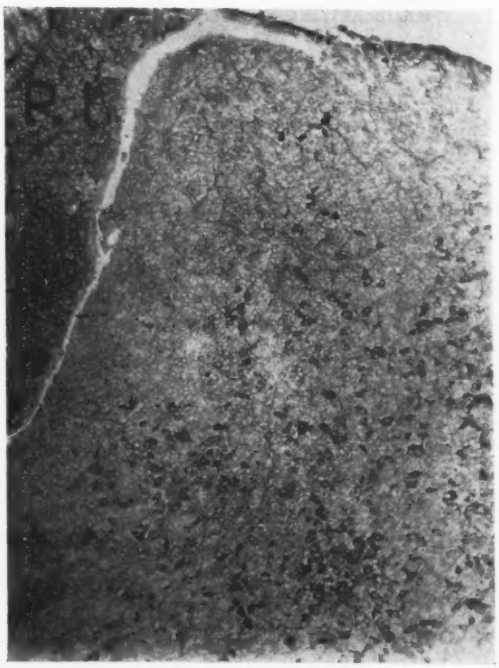

FIG. 5. Section of normal male rat pituitary stained by Gomori stain showing the absence of Gomori cells in the zone adjacent to the pars intermedia (PI).

(Gomori stain; ×120.)

Response of Gomori stained cells to thyroxine injections in thyroidectomised rats.

In order to eliminate the effect of variations in the secretions of the animals' own thyroid glands these experiments were made with thyroidectomised animals. In the first series (Table 2), the injections were started on the day after thyroidectomy and continued for thirteen days. It will be seen that 1.5 μg. of L-thyroxine prevented entirely the reduction in the numbers of Gomori positive cells which follows thyroidectomy. Injections of 3 μgms. and 5 μgms. L-thyroxine per day showed overdosage effects, the

TABLE 3. EFFECT OF L-THYROXINE INJECTIONS ON GOMORI CELLS OF THYROIDECTOMISED RATS. INJECTIONS WERE BEGUN ON THE SEVENTH DAY AFTER THYROIDECTOMY AND CONTINUED FOR SEVEN DAYS

Treatment	No. of rats	Average Gomori cells per section	
		No.	% of normal
Nil	10	0	0
0.5 μg.	2	0	0
1.0 μgs.	2	137	13
1.5 μgs.	2	220	21
2.0 μgs.	2	461	44
2.5 μgs.	2	802	76

numbers of Gomori positive cells being depressed to about 50% and 30% of normal respectively.

In another series (Table 3), the effect of thyroxine injections started one week after thyroidectomy was tested. This experiment shows the effect of thyroxine injections in restoring the Gomori staining granules after they have been completely lost as the result of thyroxine deficiency. The results show that thyroxine injections given after degranulation of the Gomori positive cells are less effective in restoring the granules than they were in retaining the normal granulation when given immediately after thyroidectomy. The maximum dosage given, 2.5 μgms. L-thyroxine per day, restored Gomori stained cells to about 80% of the normal level.

Effect of thyroxine injections on normal rats.

The effect of increasing the thyroxine supply in normal rats by daily injections of L-thyroxine was tested and the results are shown in Table 4. After fourteen days treatment with 0.5 μgm. L-thyroxine per 100 gms. per day, the Gomori cells seem to be increased in numbers but this result is not significant because of the small numbers of animals examined. 1.5 μgms. per day, approximately equivalent to the normal thyroxine re-

quirement of our rats, produced only a slight diminution in the numbers of Gomori cells though it would be expected that this dosage would almost inhibit the secretion of thyrotrophic hormone. With higher doses, overdosage effects were produced. Thus 2.5 μgms. of thyroxine per day, which is 1 μgm. per day more than the normal requirement reduced the Gomori cells to about 13% of normal and 5 μgms. per day reduced them to about 4% of the normal number. With sufficient thyroxine dosage given over a long enough time Gomori cells are completely removed from the rat pituitary. Similar results were observed in animals treated for three weeks with

TABLE 4. EFFECT OF L-THYROXINE INJECTIONS ON GOMORI CELLS OF NORMAL RATS. THYROXINE WAS INJECTED DAILY FOR FOURTEEN DAYS

Dosage of L-thyroxine per 100 gms.	Days treated	No. of rats	Average Gomori cells	
			No.	% of normal
Nil	—	2	1425	100
0.5 μg.	14	2	1703	120
1.5 μgs.	14	2	1151	81
2.5 μgs.	14	2	192	13
5.0 μgs.	14	2	63	4
5.0 μgs. increased to 10.0 μgs.	14 14	6	Nil	0

thyroid substance in doses containing 25 μgms. of organically bound iodine per 100 gms. per day.

While the number of animals examined has been small the responses to thyroxine injections have been regular. The average variation between similarly treated pairs for the animals whose pituitaries showed more than 300 Gomori cells per section was 6% of the mean value corresponding to coefficient of variation ±4.3%. However, the counts in untreated normal animals have been more variable and are possibly related to the varying states of thyroid activity in different animals.

DISCUSSION

The results of the restaining procedure showed that the Gomori stain stains specifically the glycoprotein granules in those cells which on previous evidence were considered to be purely thyrotrophic in function (Purves and Griesbach, 1951b). Apart from their functional responses, these cells can be identified from their distribution in the pituitary and by morphological characteristics. They are situated throughout the centre of the anterior lobe of the pituitary, avoiding the narrow zone adjacent to the pars intermedia which is especially rich in gonadotrophic cells. They are also

differentiated from the gonadotrophic cells by their angular outline. On the other hand the round or oval glycoprotein containing cells which are situated at the anterior edge of the pituitary adjacent to the pars intermedia, and which from their responsiveness to fluctuations in oestrogen supply are considered to be gonadotrophic in function, do not show any trace of Gomori staining granules.

Our findings of the response of the Gomori stained cells to thyroxine deficiency confirm the results of Halmi, who found that the Gomori positive cells disappeared within a few days of thyroidectomy. This loss of staining power is considered to be due to the discharge of the thyrotrophic hormone, since under the conditions of thyroxine deficiency a rapid secretion of this hormone occurs. Assays of the pituitaries of thyroxine deficient rats have shown that the thyrotrophic hormone content of such pituitaries is less than normal. However, there is a discrepancy between results of such assays and the results of the Gomori staining, since the Gomori stained granules fall to practically zero levels while the thyrotrophic hormone content, though lower than normal, is still significant (Griesbach and Purves, 1943). While, therefore, evidence indicates that the Gomori stain is staining glycoprotein granules which represent the thyrotrophic hormone in normal pituitaries, the Gomori stain does not give a staining reaction proportional to the thyrotrophic hormone content of the pituitaries of thyroxine deficient animals. It would, therefore, appear that the Gomori stain stains only the larger granules of thyrotrophic hormone in the pituitary cells, which represent stored hormone. The glycoprotein in thyrotrophic cells which is revealed by the PAS method in pituitaries from thyroxine deficient animals is either diffusely distributed or in the form of fine granules. In this form the glycoprotein is not stained by the Gomori stain.

The fact that injections of 1.5 μgs. L-thyroxine per 100 gms. body weight per day prevent any changes in the Gomori staining of the rat pituitary after thyroidectomy confirms previous findings based on Mallory staining of the rat pituitary "basophils" in indicating that the normal thyroid secretion of the rat is equivalent to 1.5 μgs. L-thyroxine per 100 gms. body weight. The effect of higher doses in reducing the number of Gomori positive cells is in accordance with the results of thyrotrophic hormone assays which show that such treatment reduces the thyrotrophic hormone content. Thyroxine in sufficient dosage for sufficient time reduces the thyrotrophic hormone content of the rat pituitary to zero, and in such pituitaries Gomori positive granules are absent.

The effect of thyroxine injections beginning one week after thyroidectomy in restoring the Gomori stained granules requires only one com-

ment. It will be seen that 1.5 μgs. of L-thyroxine per 100 gms. per day did not during the period of injection (7 days) restore the Gomori granules to normal levels. This result is probably due to the fact that a longer period of injection would be necessary to build up the thyroxine content of the animal to normal levels after it has been depleted. If the thyroxine content of 100 gms. rat were approximately 7 μgs. and the rate of thyroxine destruction about 1.5 μgs. per day, injections of 2.5 μgs. daily for seven days would approximately restore normal thyroxine levels. With this dosage the Gomori stained cells were found to be approaching normal levels after seven days.

Halmi suggested that the Gomori stained cells of the rat pituitary might be concerned with ACTH secretion since he considered that the gonadotrophic and thyrotrophic functions were carried on by the non-Gomori staining cells. The demonstration that the Gomori positive granules are glycoprotein in nature and are correlated with the content of thyrotrophic hormone in storage form makes it unlikely that the Gomori positive granules will be correlated with either content or rate of secretion of ACTH. It should be noted that Smelser (1944) assayed the central and peripheral zones of the bovine anterior pituitary gland for the thyrotrophic, adrenotrophic and gonadotrophic hormones. Although these hormones were found in greater concentration in the "basophilic" centre zone, the ratios of centre to cortex were not the same for the three hormones. From this observation he drew the conclusion that the thyrotrophic, adrenotrophic and gonadotrophic hormones of the beef pituitary gland were produced or stored by several distinct cell types which have different spacial distributions in the gland. The minor changes in the Gomori granule content which have been observed under conditions which alter the rate of secretion of ACTH can be due to a simultaneous variation in the amount of stored thyrotrophic hormone. The prompt and complete removal of Gomori positive granules by variation in the thyroxine supply shows that these granules are much more directly related to thyrotrophic hormone secretion than to ACTH.

SUMMARY

By a restaining procedure it has been demonstrated that the granules stained by the Gomori stain in the rat pituitary are glycoprotein in nature. The Gomori positive cells are identical with those "basophils" to which thyrotrophic function has been ascribed on the basis of correlation of glycoprotein content with content of thyrotrophic hormone. The Gomori positive granules are rapidly discharged in thyroxine deficiency conditions and are lost, though less rapidly, on treatment with excessive thyroxine. On the evidence of the glycoprotein nature of the Gomori granules, and the

correlation between their presence and the state of thyrotrophic function of the rat pituitary, it is considered that the Gomori stained granules are thyrotrophic hormone in a storage form. There is no evidence to relate the Gomori positive cells to adreno-corticotrophic secretion.

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THE EFFECT OF EPINEPHRINE AND INSULIN ON THE PLASMA POTASSIUM LEVEL

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INSULIN, epinephrine and desoxycorticosterone acetate have each been reported as affecting the level of blood potassium. In spite of the generally conceded fact that desoxycorticosterone is not a natural hormone of the adrenal cortex much emphasis has been placed on the role of this steroid in attempts to explain potassium homeostasis in the organism (Sayers, 1950). This status of desoxycorticosterone is the result of evidence in the literature that the level of blood potassium is increased after adrenalectomy and that plasma potassium is significantly lowered following administration of desoxycorticosterone probably by inhibiting potassium reabsorption in the kidney tubules (Harrison and Darrow, 1939; Swingle and Remington, 1944; Rommelt, Sartorius and Pitts, 1949). Evidence that epinephrine and insulin induced a rapid and significant fall in plasma potassium level in man and animals has been reported by several investigators (Houssay *et al.*, 1937; D'Silva, 1937; Flock *et al.*, 1939; Keys, 1938a, 1938b; Castleden, 1938; Moglia, 1939; Gerschman, 1939; Brewer *et al.*, 1939).

In view of the recent evidence that epinephrine (Vogt, 1944; Long and Fry, 1945; Long, 1947) and insulin (Vogt, 1947; Gershberg and Long, 1948; Dury, 1950) via "reflex" stimulation of the adrenal medulla induced increased adrenocortical activity, it appeared that the regulation of the plasma potassium level by at least three different humoral agents (as described in the literature) required further investigation. It is therefore possible to ascribe the decreased level of plasma potassium which followed the administration of epinephrine or insulin as resulting from: 1. The physiological action of increased secretion of adrenocortical steroids which are desoxycorticosterone-like in activity; 2. The direct physiological action of both epinephrine and insulin as such; 3. The effect of either epinephrine or insulin alone since in the normal animal the administration of either substance has been shown to "reflexly" release the other humoral agent (Cannon *et al.*, 1924); or 4. The effect of hyper- or hypoglycemic blood levels, per se, on the level of plasma potassium consequent to the administration of epinephrine or insulin.

Received for publication Sept. 20, 1950.

It is the purpose of this paper to present evidence that a significant decrease in the level of plasma potassium was induced with epinephrine which did not involve the mediation of the adrenal cortex; that insulin's apparent effect on the plasma potassium level in normal intact rats was mediated via the adrenal medulla; and that the blood glucose level as such does not have any effect on the level of plasma potassium.

MATERIALS AND METHODS

Normal intact, adrenal enucleated,[1] adrenalectomized and hypophysectomized[2] groups of rats weighing approximately 200 grams were used in this investigation. They were fed a chow biscuit and water ad libitum (the demedullated and adrenalectomized rats were given a 1% NaCl drinking solution) supplemented with greens once weekly. The demedullated and adrenalectomized rats were used in the experiments when 14–16 days after their surgery. The hypophysectomized rats were used 3–4 days after their surgery. Groups of normal intact, adrenal medullectomized, adrenalectomized and hypophysectomized rats were subjected to one of the following procedures: *Physiological saline* (0.5 ml., sub-q) controls; *Epinephrine* (0.02 mg./100 gm. rats, sub-q); *Insulin* (ILETIN, regular, diluted fresh with physiol. saline, 0.5 *U*/rats, sub-q); and *Insulin* (0.25 *U*/rat, sub-q) followed five minutes later by epinephrine (0.02 mg./100 gm. rat, sub-q). In addition to these two groups of intact rats were given 2 ml. of 50% glucose solution per os. All of the injections and the glucose per os were administered to the rats immediately after they had been lightly anesthetized with EVIPAL (n-methylcyclohexenyl-methyl-barbituric acid). Sixty or 180 minutes after the administration of the different agents blood was obtained by cardiac puncture from the rats after having been lightly anesthetized again with EVIPAL. The blood was immediately centrifuged and aliquots of the plasma (if completely clear of hemolysis) were taken for the determination of plasma potassium, glucose and sodium (if sufficient sample was available). The plasma levels of potassium and sodium (m.eq./L) were determined with an internal lithium standard flame photometer. The plasma glucose levels (mg.%) were determined by a photometric modification (Kingsley and Reinhold, 1949) of the method of Folin and Wu. The difference between the mean plasma potassium levels (m.eq./L) of each of the experimental groups 60 or 180 minutes after the administration of the experimental agent and their respective controls (physiological saline-injected) was tested for statistical significance.

The periods of 60 and 180 minutes after the administration of the different experimental agents were selected to measure the effect of these agents on the level of plasma potassium in view of the following lines of evidence in the literature concerning the relationship of epinephrine, insulin and the adrenal cortex: Castleden (1938) reported a maximum fall in the plasma potassium level induced in man approximately 20 minutes after epinephrine injection. In this laboratory we have also induced a maximum fall in plasma potassium approximately 20 minutes after epinephrine injection in normal humans and

[1] Dr. Leo Moss, Pathologist, Bradford Hospital studied the histological sections of the regenerated adrenal cortices of this group of rats and confirmed the absence of adrenal medullary tissue and the histological regeneration of the three zones of the adrenal cortices.

[2] The rats were hypophysectomized by the Hormone Assay Laboratories Inc., Chicago, Ill.

a maximum fall in potassium after 60 minutes in Addisonian patients (unpublished data). The rapid hypoglycemic action of regular insulin and the "reflex" stimulation of the secretion of the adrenal medulla is well known (Cannon *et al.*, 1924). In recent years evidence has been presented that epinephrine and insulin induced increased adrenocortical activity 60 and 180 minutes after their injections and that insulin's effect was mediated via "reflexly" stimulating the adrenal medulla (Long and Fry, 1945; Vogt, 1947; Dury, 1950). Cognizance of these interrelationships of epinephrine, insulin and adrenocortical activity and the time elements involved determined the design of the experiments reported here.

RESULTS

The effect of epinephrine, insulin or insulin plus epinephrine 60 minutes after the injection of these agents upon the level of plasma potassium is

TABLE 1. THE REDUCTION IN THE PLASMA POTASSIUM LEVEL (M.EQ./L) 60 MINUTES AFTER EPINEPHRINE OR INSULIN INJECTIONS

Experimental Animal	Physiol. Saline (0.5 ml., s.c.) Plasma		Epinephrine, s.c. (0.02 mg./100 gm.) Plasma		Insulin, s.c. (0.5 *U*/rat) Plasma		Insulin plus[1] Epinephrine, s.c. Plasma	
	K m.eq./L	Glucose mg. %	K m.eq./L	Glucose mg. %	K m.eq./L	Glucose mg. %	K m.eq./L	Glucose mg. %
Intact	4.76 ± .18[2] (12)	177 ± 8 (11)	4.21 ± .10 (8)	300 ± 9 (8)	3.81 ± .11 (13)	45 ± 8 (6)	3.73 ± .06 (6)	70 ± 6 (7)
P =			0.03[3]		0.001		0.001	
Adrenx	4.95 ± .21 (9)	80 ± 8 (8)	3.58 ± .08 (7)	185 ± 16 (7)	4.58 ± .12[4] (9)	20 ± 2 (10)	3.39 ± .05 (7)	52 ± 10 (7)
P =			0.001				0.001	
Adrenal Demed.	3.49 ± .12 (7)	80 ± 6 (7)	2.55 ± .05 (8)	243 ± 13 (8)	3.62 ± .12 (5)	27 ± 3 (5)	2.76 ± .14 (5)	45 ± 4 (6)
P =			0.001				0.003	

[1] Insulin (0.25 *U*/rat) followed 5 min. later by epinephrine (0.02 mg./100 gm. rat), s.c.
[2] Mean ± S.E.
[3] Comparison with physiological saline injected group.
[4] The insulin dose in this group was 0.25 *U*/rat, s.c.
The number in parentheses is the number of rats in the group.

presented in Table 1. Inspection of these data show that a statistically significant fall in the level of plasma potassium was induced in the groups of normal intact rats after each of the procedures. In the groups of adrenalectomized and adrenal enucleated rats epinephrine alone or the combination of insulin plus epinephrine also significantly lowered the mean level of plasma potassium. Insulin alone, however, in these groups had no effect on the plasma potassium level. It is apparent from these results that epinephrine's effect on the plasma potassium level was not mediated by the adrenal cortex whereas insulin's effect on the plasma potassium in the intact rats was mediated via the adrenal medulla.

The data in Table 2 summarize the results obtained 180 minutes after epinephrine, insulin or insulin plus epinephrine injections on the mean level of plasma potassium in normal intact, adrenalectomized, and adrenal

TABLE 2. EFFECT OF EPINEPHRINE OR INSULIN ON THE PLASMA POTASSIUM LEVEL (M.EQ./L) 180 MINUTES AFTER INJECTION

Experimental Animal	Physiol. Saline (0.5 ml., s.c.) Plasma		Epinephrine, s.c. (0.02 mg./100 gm.) Plasma		Insulin, s.c. (0.5 *U*/rat) Plasma	
	K m.eq./L	Glucose mg. %	K m.eq./L	Glucose mg. %	K m.eq./L	Glucose mg. %
Intact	4.28∓.18[1] (10)	114∓16 (8)	4.21∓.20 (8)	223∓17 (5)	3.73∓.23 (8)	42∓6 (6)
Adrenx	4.59∓.27 (5)	95∓8 (6)	4.57∓.10 (4)	233∓37 (4)	[2]	
Adrenal Demed.	4.13∓.14 (8)	88∓5 (9)	3.52∓.17 (7)	337∓9 (7)	3.84∓.09 (11)	47∓7 (4)
P =			0.01[3]			

[1] Mean ± S.E.
[2] The animals in this group were moribund.
[3] Comparison with physiological saline injected group.
The number of rats in the group is in the parentheses.

demedullated groups of rats. Only in the demedullated group after epinephrine was a significant fall in the mean level of plasma potassium obtained. An explanation for this particular result is not apparent from the available data. The results presented in Tables 1 and 2 demonstrate that

TABLE 3. PLASMA SODIUM LEVELS (M.EQ./L) IN GROUPS OF RATS 60 AND 180 MINUTES AFTER EPINEPHRINE OR INSULIN

Experimental Animal	Physiol. Saline (0.5 ml., s.c.)	Epinephrine, s.c. (0.02 mg./100 gm.)	Insulin, s.c. (0.5 *U*/rat)
60 minutes after injections			
Intact	146[1](8) 144–150[2]	144.8 (5) 142.8–146.4	147.4 (7) 141.6–151.8
Adrenx	147 (2)	144 (4) 135–152.4	—
Adrenal Demed.	147.9 (8) 146–150	145.7 (5) 144.4–147	151 (2)
180 minutes after injections			
Intact	145.6 (9) 140.8–147.2	144.9 (4) 141–148.2	148.3 (4) 144.6–153
Adrenx	147.8 (3) 144.6–151.2	—	—
Adrenal Demed.	149.7 (6) 145.8–154.2	145.4 (6) 139.8–150.9	149.6 (10) 147–155.4

[1] Mean plasma sodium level (m.eq./L).
[2] Range of plasma sodium values (m.eq./L) in the group.
The number of rats in the group is in the parentheses.

the epinephrine effect on the mean level of plasma potassium was one of rapid change and then adjustment by the organism.

The mean plasma sodium levels and range of values (m.eq./L) for the groups of rats 60 and 180 minutes after physiological saline, epinephrine or insulin are given in Table 3. Since an aliquot of plasma for sodium determination was not available for some of the groups and the number of determinations in some of the groups were small, these results were not considered suitable for statistical testing. Inspection of the data indicate however that the plasma sodium level was not affected by the experimental procedures. The wide range of plasma sodium levels reported here is

TABLE 4. THE LACK OF EFFECT OF HYPERGLYCEMIA, PER SE, ON THE LEVEL OF PLASMA POTASSIUM (M.EQ./L) IN INTACT RATS

Experimental Animal	60 Min. after Agent Plasma		180 Min. after Agent Plasma	
	K m.eq./L	Glucose mg. %	K m./eq./L	Glucose mg. %
Physiol. saline (0.5 ml., s.c.)	4.76∓.18[1] (12)	177∓8 (11)	4.28∓.18 (10)	114∓6 (8)
50% Glucose (2 ml. per os)	4.53∓.15 (11)	224∓7 (11)	4.02∓.15 (5)	169∓5 (5)

[1] Mean ±S.E.
The number of rats in the group is in the parentheses.

apparently not uncommon. Brownell, Hartman and Reiman (1950) also reported such ranges in normal and demedullated rats and commented upon conditions which might be related to such findings. They concluded that "rats maintain their serum sodium level very poorly."

Since epinephrine induces a hyperglycemia, it appeared essential to determine whether the fall in plasma potassium level 60 minutes after epinephrine injection (Table 1) was related to the elevated blood glucose. The effect of the administration of 2 ml. of 50% glucose solution per os on the plasma potassium level after 60 and 180 minutes in normal intact rats is shown in Table 4. The results in these groups clearly show that hyperglycemia as such did not affect the level of plasma potassium. The results obtained in the groups of rats injected with insulin plus epinephrine (Table 1) are also relevant to this question of the relationship of the blood glucose level to the change in plasma potassium. In these groups of rats a significant depression in the level of plasma potassium was induced although the animals were definitely hypoglycemic. It is apparent from these results that the level of blood glucose was not an integral part of the mechanism affecting the level of plasma potassium. Epinephrine, however, appears to

be clearly responsible for the depression induced in the plasma potassium level.

Since epinephrine is a recognized stimulating agent of the anterior pituitary, the effect of epinephrine on the level of plasma potassium was tested in the hypophysectomized rat. Inspection of the data presented in Table 5 show that epinephrine induced a statistically significant depression in the mean level of plasma potassium 60 minutes after its injection. Measurement of the adrenal ascorbic acid concentration by the method of Roe and Keuther (1943) showed no change in the hypophysectomized rats 60 min-

TABLE 5. THE EFFECT OF EPINEPHRINE ON THE PLASMA POTASSIUM LEVEL IN HYPOPHYSECTOMIZED RATS

Experimental Animal	60 Minutes after Physiol. Saline, s.c.[1]		60 Minutes After Epinephrine, s.c.[2]	
	Plasma K m.eq./L	Ascorbic acid mg./100 gm. adrenal	Plasma K m.eq./L	Ascorbic acid mg./100 gm. adrenal
Hypophysect.[3]	5.32 ± .12 (5)	404 ± 15 (5)	4.63 ± .22 (7)	377 ± 19 (4)
P =			0.03[4]	
Normal intact	4.76 ± .18 (12)	360 ± 17 (12)	4.21 ± .10 (8)	239 ± 7 (4)
P =			0.03	

[1] 0.1 ml./100 gm. rat.
[2] 0.02 mg./100 gm. rat (0.1 ml.).
[3] Sprague-Dawley strain hypophysectomized by Hormone Assay Labs. Inc. All other rats are Wistar strain.
[4] Comparison with physiological saline injected group.
The number in parentheses is the number of rats in the group.

utes after epinephrine. Since this dose level of epinephrine induced a significant fall in the plasma potassium level and the concentration of adrenal ascorbic acid 60 minutes after injection in the normal rat, the results presented in Table 5 provide additional evidence that this dose of epinephrine induced a significant fall in plasma potassium which was not mediated through the anterior pituitary-adrenocortical axis.

DISCUSSION

Cori (1931) pointed out that the process of esterification of inorganic phosphate resulting in an increase in muscle hexosemonophosphate was an epinephrine response. The similar effect following insulin was suggested as resulting from a reflex stimulation of epinephrine consequent to insulin hypoglycemia. It is known that potassium has an essential role in carbohydrate metabolism in the esterification of inorganic phosphorous (Fenn, 1940; Gass *et al.*, 1948). The observations (see Introduction) that plasma

potassium was lowered after insulin injection in animals and man has led to the acceptance that insulin in normal animals specifically affects the level of plasma potassium. It is evident from the results presented here that insulin, per se, did not affect the plasma potassium level but that the adrenal medulla mediated this apparent insulin effect on plasma potassium in the normal intact group of rats. The results following epinephrine injection indicate that this humoral substance as such, and not involving the pituitary-adrenocortical axis, should be considered as having an integral part in the mechanism of potassium homeostasis. Recently Rogoff, *et al.* (1950) reported evidence which supports this thesis. They showed a significant fall in plasma potassium was induced in an adrenalectomized dog given an infusion of a physiological level of epinephrine.

SUMMARY

Epinephrine (0.02 mg./100 gm. rat, s.c.) was shown to induce a significant fall in the mean level of plasma potassium in normal intact, adrenalectomized, adrenal enucleated and hypophysectomized groups of rats 60 minutes after injection. There was no change in the level of plasma potassium in normal or adrenalectomized groups of rats 180 minutes after epinephrine injection.

Insulin (0.5 *U*/rat, s.c.) induced a significant fall in the mean plasma potassium level in normal intact rats 60 minutes after its injection, but did not affect the plasma potassium in the groups of adrenalectomized or adrenal enucleated rats. Nor did insulin affect the plasma potassium in any of these groups of rats 180 minutes after its injection.

Evidence is presented that epinephrine as such should be considered as having an integral role in potassium homeostasis which does not involve the anterior pituitary-adrenocortical axis. Insulin's effect on the level of plasma potassium in normal intact rats is shown to be mediated by "reflex" stimulation of the adrenal medulla. Data were presented that the level of blood glucose, per se, did not affect the plasma potassium level.

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NOTES AND COMMENTS

FAILURE OF CORTISONE TO REDUCE ADRENAL WEIGHT IN HYPOPHYSECTOMIZED RATS

Severe adrenal cortical atrophy in rats has been described after massive doses of cortin (Ingle and Kendall, 1937; Ingle, *et al.*, 1938; Ingle, 1938) and of crystalline cortisone (Ingle and Mason, 1938; Wells and Kendall, 1940; Stebbins, 1950; Winter, *et al.*, 1950). This atrophy was prevented when ACTH and cortin were administered together (Ingle, *et al.*, 1938). In hypophysectomized rats treated simultaneously with cortin and ACTH, adrenal cortical atrophy also failed to occur (Ingle, 1938). This has been interpreted to mean that atrophy of the adrenal cortex may be due to a restricted output of adrenotrophin (Ingle, 1938). In further support of this hypothesis is the evidence that the increased secretion of adrenotrophin during stress is suppressed by cortisone (Sayers and Sayers, 1949).

In the present study, it was observed that the injection of large amounts of cortisone (17-hydroxy-11-dehydrocorticosterone acetate) to hypophysectomized rats did not significantly influence the weight of the adrenal gland.

METHODS AND MATERIALS

Twenty-four adult male rats of the Sprague-Dawley strain were grouped as indicated in Table 1. These animals were hypophysectomized and maintained for 1 week to overcome the effects of the operation. The rats were housed in a warm draft-free environment and fed a mixed diet. Water was supplied *ad libitum*. Three mg. of Cortone (Merck), a saline suspension of micronized material, was administered subcutaneously once daily to twelve animals. Each control rat was paired by weight with a partner from the steroid-treated group. This was accomplished by restricting the food intake of the control so that its daily body weight change approximated that of its Cortone-treated partner. The animals were sacrificed by exsanguination 24 hours after the last dose. Group 1 was continued under treatment for 10 days; group 3 for 30 days.

RESULTS

Average adrenal and body weights are presented in Table 1. The data show that the

TABLE 1. CORTISONE IN HYPOPHYSECTOMIZED RATS

Group	Treatment	No. rats	Gm. Initial body weight	Gm. Final body weight	Gm. adrenal weight
1	Cortisone 3 mg./day for 10 days	6	177	142	.015 (.0142–.0160)
2	Pair-weight Controls	6	176	146	.015 (.0145–.0156)
3	Cortisone 3 mg./day for 30 days	6	188	150	.010 (.0093–.0108)
4	Pair-weight Controls	6	187	148	.010 (.0094–.0106)

Received for publication December 4, 1950.

size of the adrenal gland is the same in the animals of groups 1–4. The weights of the adrenal glands of these hypophysectomized rats are about 50% below that of the intact rat.

CONCLUSION

The administration of Cortone to hypophysectomized rats produced no greater atrophy of the adrenal gland than that due to hypophysectomy alone. This finding is consistent with the thesis advanced by others that Cortone produces atrophy of the adrenal cortex not by direct action upon the adrenal, but by suppression of the release of adrenotrophin of the pituitary gland.

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A STUDY OF THE RAT VENTRAL PROSTATE GLAND IN TISSUE CULTURE[1,2]

It has long been known that the ventral prostate of male rats castrated soon after birth continues to grow and differentiate, and ultimately reaches, for a limited time, a histological condition that is similar to that of a gland in a normal male (Price, 1936). This development includes the enlargement of the acini, the formation of lumina in the acini and the development of the cellular light areas which are typical of cells in the functioning gland.

The growth and development of the prostate in the castrated male have been attributed to the stimulation of the gland by adrenal androgen (Howard, 1937, 1938) and experimental evidence supports this contention (Burrill and Greene, 1939, 1940). An analysis of the problem by means of prostate transplants indicated that the gland does not self differentiate (Price, 1941). However, the possibility that the prostate gland is ca-

Received for publication June 25, 1951.

[1] Through the kindness of Dr. Honor B. Fell the facilities of The Strangeways Laboratory were placed at my disposal. I am indebted to her for help, advice and interest, in this preliminary study.

[2] This research was aided by a grant from the Joseph Henry Fund of the National Academy of Sciences and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of The University of Chicago.

pable of some degree of self-differentiation must still be considered. The present comn.unication presents some data from a study of the gland in tissue culture.

MATERIALS AND METHODS

Ventral prostate glands were dissected from 6-day-old hooded rats and cultured by the watch glass method (Fell, 1940) on a medium composed of ½ cock blood plasma and ½ saline extract of chick embryos of 13 days incubation. The gland was separated, in most cases, into right and left lobes which were explanted separately and cultured for 7, 11, or 25 days, with a change to fresh medium every third day. At the end of the culture period the explants were fixed in Zenker's or Bouin's fluid and prepared for histological study. Eleven prostate explants were studied and compared with glands from 6-day-old rats.

RESULTS

Ventral prostate explants survived well in watch glass cultures. All the explants were similar in that development had not progressed after explantation, although the explants survived without necrosis, and mitotic activity was going on in the epithelium of the acini. Most of the explants were practically indistinguishable histologically from normal 6-day-old tissue. The acini were approximately the same diameter in the explant as at the time of explantation and the formation of lumina in the acini had not progressed although the explants appeared normal even after a culture period of 25 days. This suggests that hormone stimulation may be essential for development. The problem is being further analyzed by the use of different media and by the introduction of hormones into the media.

SUMMARY

Rat ventral prostate glands were cultured by the watch glass method. The explants survived very well, appeared essentially normal histologically and showed mitotic activity. The tissue did not develop beyond the 6-day-old stage at which it was explanted. The results suggest that even the early stages of prostate development may be hormone-conditioned.

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THE EFFECT OF THE INGESTION OF A HYPOLIPOTROPIC DIET ON THE PLASMA PSEUDO-CHOLINESTERASE ACTIVITY OF SPAYED FEMALE RATS

It has been shown previously that the ingestion of a diet low in lipotropic factors induces a highly significant elevation in the plasma pseudo-cholinesterase activity of adult and weanling male rats (Hawkins and Nishikawara, 1951). This elevation is not observed when supplements of choline, betaine or methionine are added to the basal diet. The triethyl homologue of choline is not as efficient as these compounds either in lowering liver lipids or in preventing the rise in the pseudo-cholinesterase activity.

In female rats, on the other hand, the ingestion of a hypolipotropic diet leads to a significant depression in the plasma pseudo-cholinesterase activity. The level of this enzyme in the plasma and liver of female rats is four to five that in the same tissues of male rats (Zeller and Birkhauser, 1941; Beveridge and Lucas, 1941; Mundell, 1944). The higher level of estrogen in the female rat has been shown by Sawyer and Everett (1946) and Everett and Sawyer (1946) to be responsible for this sex difference in pseudo-cholinesterase activity.

In the present investigation spayed female rats were offered a hypolipotropic diet to ascertain whether a depression in the estrogen level of these animals would modify the response of their plasma pseudo-cholinesterase to this diet.

METHODS

Twenty-four adult female Wistar rats (140–170 gm.) were spayed and divided into 2 groups, 0 and I. A third group, II, of comparable weight was sham-operated. The animals were allowed to recover postoperatively for one week. During this period they were fed a diet of fox chow ad libitum. For the following 4 weeks, group 0 was offered ad libitum the basal hypolipotropic diet (see Hawkins and Nishikawara, 1951) and groups I and II were given the same diet supplemented with 0.35% choline chloride. The latter groups were "pair-fed" against the former.

The animals were sacrificed under light ether anesthesia by exsanguination through the carotid artery. The blood was collected in the presence of a small amount of sodium oxalate, centrifuged immediately and the plasma used for pseudo-cholinesterase estimations. Gross examination of the uteri of the spayed animals at this time revealed marked atrophy in comparison with those of the sham-operated females.

Enzymatic activity was measured manometrically in a medium of 0.025 M. $NaHCO_3$ saturated with 5% CO_2 in N_2 (pH 7.4). The substrate employed for pseudo-cholinesterase estimations was 0.006 M. benzoylcholine (Mendel, Mundell and Rudney, 1943).

RESULTS

The figures in Table 1 show that spaying induces a significant depression in the plasma pseudo-cholinesterase activity of female rats. This observation is in agreement with those of Sawyer and Everett (1946). However, when spayed rats were maintained on a hypolipotropic diet, the activity of the enzyme in their plasma was significantly elevated over that of the "pair-fed" spayed controls consuming a normal (choline-containing) diet. This is in contrast to results obtained in normal female rats where a significant depression in the plasma pseudo-cholinesterase results from the ingestion of the hypolipotropic diet. Thus when the estrogen level is low, an elevation rather than a depression of this enzyme results when the dietary supply of lipotropic substances is inadequate.

SUMMARY

In contrast to the depression observed when intact female rats are maintained on a diet low in lipotropic factors, a highly significant elevation in the plasma pseudo-cholin-

Received for publication June 28, 1951.

esterase activity occurs in spayed animals. The ingestion of this diet induces a similar elevation in the enzyme of the plasma of male rats. The sex difference in the response of rats to the hypolipotropic diet would seem, therefore, to be due to the difference in their estrogen levels.

TABLE 1. THE EFFECT OF ESTROGEN LEVELS ON THE PLASMA PSEUDO-CHOLINESTERASE ACTIVITY[1] OF RATS MAINTAINED ON A HYPOLIPOTROPIC DIET

	Females			Males[2]	
Treatment	Sham-operated	Spayed	Spayed	—	—
Diet	+choline	+choline	Basal	+choline	Basal
Enzyme Activity	125.3±8.5[3]	76.9±6.3	120±10.7	36.2±2.7	79.8±4.5
No. Rats	10	11	12	20	20
"Students" t value	4.64	3.47		6.7	
P	<0.01	<0.01		<0.001	

[1] μl. CO_2 evolved in 20 minutes.
[2] See Hawkins and Nishikawara (1951).
[3] Mean ± standard error.

Acknowledgements

The author wishes to thank Doctors C. C. Lucas and R. D. Hawkins for helpful criticism and advice.

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